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Transplantation of Leukemia from Inbred to Noninbred Guinea Pigs[1, 2]

ELI M. NADEL,[3] *M.D.*, *National Cancer Institute,*[4] *Bethesda, Maryland*

The guinea pig recently has been shown to be a particularly suitable animal for the study of urinary corticosteroid excretion (*1–6*). Because of considerable interest in the possible effect of leukemia on the pattern of corticosteroid excretion, it was highly desirable to be able to study leukemia experimentally in the guinea pig. The problem was beset with difficulties, for although the techniques for the isolation and quantitative measurement of corticosteroids from the urine of guinea pigs had now been developed (*1–6*), a source of leukemia for study in this species was not accessible.

Not only were there no known colonies of guinea pigs with a predictable and useful incidence of spontaneous leukemia but also no useful technique for producing leukemia in guinea pigs was known. Although a few cases of spontaneous leukemia were recently reported in highly inbred (strain 2; strain 13) guinea pigs (*7*), it was believed that highly inbred animals were needed for successful transplantation studies, and sufficient numbers of these inbred animals were not available for the experimental study contemplated.

An attempt was made to transfer 1 of the spontaneous leukemias, known as acute lymphocytic leukemia 2B (L2B), from its highly inbred strain of origin (strain 2) to stock, noninbred guinea pigs. This attempt at homoiotransplantation was successful in very young guinea pigs (*8*) and is herein reported.

Experimental

Materials

Leukemia 2B.—L2B is a lymphocytic leukemia that arose in a strain 2 female guinea pig slightly over 30 months of age. The tumor was supplied by the late Dr. Egon Lorenz (*7*).

[1] Received for publication March 22, 1953.

[2] Presented by title as an abstract at the 39th annual meeting of the American Society of Experimental Pathology, April 15–17, 1954, in Atlantic City, N. J.

[3] Thanks are extended to Dr. C. A. Velat for permission to quote his corroborative findings. Mr. W. Stalters, Mr. A. Hilgar, and Mr. B. Young gave valuable assistance in these studies.

[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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Inbred strain 2 guinea pigs.—This highly inbred strain was developed in the Department of Agriculture through brother × sister matings beginning in 1906. The history and present maintenance of this strain has been described (*9*).

Noninbred (stock) guinea pigs.—All stock animals were pen bred and maintained by the Animal Section, Laboratory Aids Branch, National Institutes of Health. No attempt has been made to develop genetically inbred strains from the stock animals described. The names Hartley and Beltsville are used to identify the animal colonies but do not imply inbreeding in the genetic sense.

a) Hartley.—Albino guinea pigs were purchased from the Tumblebrook Farm, Brant Lake, New York, in May, 1950. The guinea pigs were descendants of a "self-white" line developed by Sir Percival Hartley, of the National Institutes of Medical Research, Hampstead, England. The degree of inbreeding present after the line left England is not known.

b) Beltsville.—Multicolored guinea pigs, so designated, were obtained from the breeding colony of the Department of Agriculture at Beltsville, Maryland, in September, 1950.

Throughout this paper the terms *strain* and *stock* are used in the sense described by Engelbreth-Holm (*10*) and by the Committee on Standardized Nomenclature for Inbred Strains of Mice (*11*). The term *strain* implies that a high degree of inbreeding, the result of over 20 consecutive generations of brother × sister or parent × offspring mating, has been achieved. The term *stock* indicates that the guinea pigs are not highly inbred.

Methods

Brei preparation.—All transplants were made from the spleens or from tumor masses of guinea pigs with leukemia, diagnosed by examination of a stained smear of the peripheral blood and confirmed by autopsy examination and histopathologic study. A weighed quantity of splenic tissue was used to prepare a brei, using Locke's solution as diluent (1:2). One cc. of the brei then was injected, either into the right axillary space or into the peritoneal cavity, in the first 2 attempts at transplantation into noninbred animals. Subsequently, ¼ to ½ cc. of spleen brei (1:1 to 1:4) was injected intraperitoneally or subcutaneously, or spleen or tumor tissue was implanted subcutaneously by trocar.

Pathologic study.—Evidence of the presence of leukemic cells in the peripheral blood was provided by smears, prepared with Wright's stain, white-blood-cell counts, or a combination of these procedures, which were performed at least once before the death of each animal. In some cases the progress of the disease was followed by additional counts and smears. A complete autopsy, including examination of the brain, was performed on each animal. Histopathologic study was based on formalin-fixed paraffin sections, stained with hematoxylin and eosin.

Animals.—A total of 178 noninbred guinea pigs and 20 strain 2 guinea

TABLE 1.—*Transplantation of leukemia 2B in noninbred and control guinea pigs*

Age at transplant (*days*)	Transplant generation	Route*	No. of animals	Progressive takes		Speed of lethality (*days*)
				No.	Percent	
Noninbred (Hartley; Beltsville)						
3–7	23–27	SC & IP	23	23	100	19. 3
4–7	28–32	IP	32	32	100	21. 3
2–4	33–37	IP	18	15	83	18. 0
5–9	38–41	SC	18	12	66	16. 4
23	23–24	IP	2	2	100	29. 4
34	25–32	SC	8	6	75	23. 2
37	23–26	IP	5	5	100	18. 2
35–45	38–41	SC & IP	48	5	11	34. 4
48	23–26	IP	12	0	0	—
7 wks.–2 yrs.	28–32	SC & IP	12	0	0	—
AVERAGE SURVIVAL						20. 7
Controls (inbred strain 2)						
(*months*)						
4–10	23–33	IP	12	12	100	16. 3
12	34–41	IP	8	8	100	17. 0
AVERAGE SURVIVAL						16. 6

*SC = subcutaneously; IP = intraperitoneally.

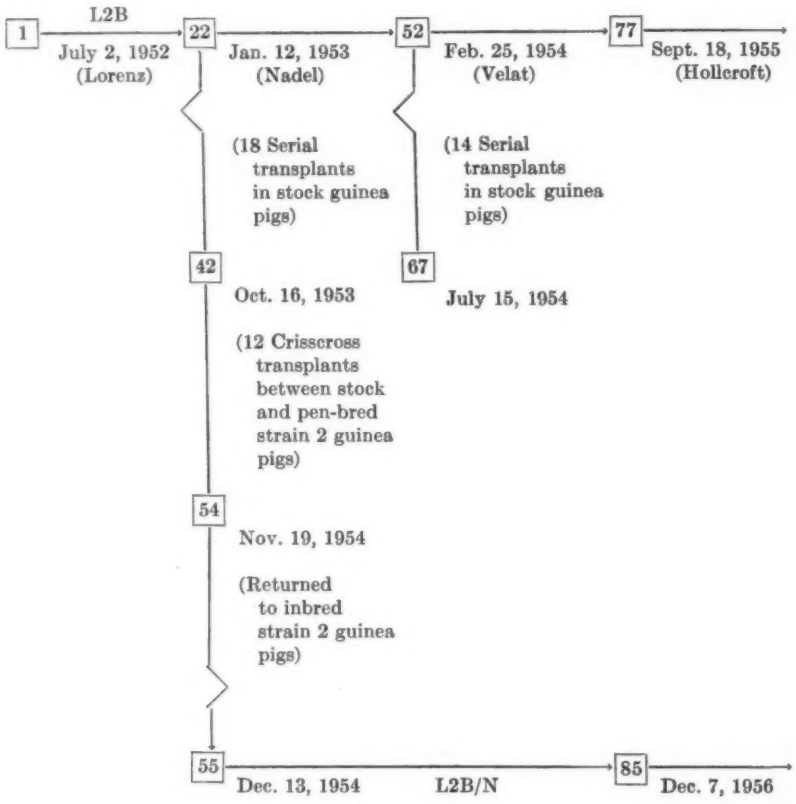
pigs received transplants from different transplant generations carried in inbred and noninbred animals. Animals were of either sex and ranged in age from 2 days to 2 years at the time of transplantation. The results of transplantation for 18 generations in noninbred animals and then in 12 crisscross generations, alternating between noninbred, stock guinea pigs and pen-bred strain 2 guinea pigs, are given in table 1. Inbred strain 2 (4–12 months old) animals received transplants during this period and served as controls of the speed of lethality of the tumor.

Transplantation procedure.—The original transplant was a spleen brei of L2B in its 22nd transplant generation in the strain 2 guinea pig. Following 2 serial transplantations outside the inbred strain of origin, the tumor was maintained in the Laboratory of Pathology, National Cancer Institute, by transplantation into noninbred 2-to-4-day-old Hartley guinea pigs for 16 more consecutive transplant generations. A few pen-bred strain 2 guinea pigs then became available, and it was possible to utilize these in 12 serial crisscross passages between noninbred, stock guinea pigs and pen-bred strain 2 animals for 12 generations, when a further increase in the supply of inbred strain 2 guinea pigs assured the passage of the tumor solely in inbred animals. The genealogy of the tumor studied is depicted in chart 1.

Results

The major morphologic findings in animals that died with L2B have been described (*7*), but clinical and pathologic features not then emphasized or described deserve some attention and are noted herein.

CHART 1.—*Guinea pig leukemia 2B (Lorenz).* □=Transplant generation number.



Clinical Findings

Each animal with a progressive take died with clinical and morphologic evidence of leukemia. Tumor masses were present at the site of injection and/or enlarged lymph nodes were palpable in all animals prior to death. Spleens were palpable in most animals 1 to 3 days prior to death. On the day of death, respiratory movements were usually labored, food was only partly eaten, and there usually was ataxia or an inability to resume an upright position after being placed on the back or side. After receiving a fresh spleen-brei transplant, the average survival time for the 100 non-inbred animals with progressive takes of leukemia was 20.7 days, with a range of 12 to 34 days, while the average survival of 20 strain 2 guinea pigs was 16.6 days, with a range of 14 to 17 days. The 78 animals without a progressive take after injection of viable tumor were maintained for 20 months in the Laboratory of Pathology, National Cancer Institute, and

following cranioconcussions were examined *post mortem*. There was no gross or histologic evidence of leukemia.

Hematologic Findings

All leukemic animals had abnormal cells in the peripheral blood prior to death and, at death, smears indicated that such cells constituted approximately 99 percent of the circulating white blood cells. A few cells resembling abnormal monoblasts were seen in an otherwise uniform population of abnormal cells resembling lymphoblasts (fig. 2). Mitotic figures in the peripheral blood were seen regularly in the terminal cases (fig. 4), while only a rare normoblast or granulocyte was found. Binuclear cells were occasionally seen—again terminally. An increase in leukemic cells in the peripheral blood was often preceded by the presence of increased numbers of basophilic granulocytes. On the 12th day after transplantation total white-cell counts in the different animals ranged from 4,250 to 950,000 cells per $mm.^3$ of blood. At least 2 animals with palpable tumors had a period of leukopenia as late as 19 days after transplantation. Of the animals on which serial white-blood-cell counts were made, all showed a progressive increase in the total counts prior to death.

Autopsy Findings

There was no loss of weight, and animals with leukemia died in good nutritional condition. Tumor nodules were present at the site of injection in all noninbred animals. In noninbred guinea pigs bearing intraperitoneal transplants, large, fleshy tumor masses regularly developed at the injection site and extended through the entire thickness of the abdominal body wall. These masses were often over 2 cm. in each diameter. In some, blood-tinged fluid filled the peritoneal cavity. This may have been due to overzealous palpation of the spleen during life. No fluid was present in the thoracic cavity of any animal. None of the strain 2 animals showed blood-tinged peritoneal fluid or tumor masses that penetrated the skin after intraperitoneal injection. Retroperitoneal, periaortic, perirenal, perihepatic, and peripancreatic lymph nodes were enlarged in all animals. The cecal and mesenteric nodes were particularly enlarged—the latter often measuring over 3 cm. in diameter (fig. 1). The liver (fig. 6) and spleen were regularly infiltrated and enlarged (fig. 1) in all animals. Leukemic spleens weighed from 3.7 to 6.8 gm. in comparison to an average of 360 mg. for normal animals of similar total body weight (fig. 1). In some guinea pigs, focal hemorrhagic areas were present in the cerebellum (fig. 3) and tumor thrombi were present in the 4th ventricle. Hemorrhages were not present in the cranial cavity in any of the animals, except within the brain substance, as mentioned. Leukemic infiltrations were regularly present in the adrenal glands (fig. 5). Adrenals often contained punctate areas of hemorrhage and usually were depleted of lipides. Petechial hemorrhages were often present in the skin and on the parietal peritoneal surface. Cervical nodes were often enlarged in continuity with axillary

nodes. Peyer's patches were prominent, elevated, and hyperemic in all animals.

Microscopic Findings

Histopathologic study confirmed the hematologic and gross autopsy diagnoses. All animals died with an acute form of lymphocytic leukemia. Local tumor formation was present at the site of injection and infiltrations of leukemic cells were widely distributed throughout the body tissues (figs. 1–6). Histologically, the growth of leukemia 2B in stock guinea pigs was similar to that in strain 2 animals (*7*).

Transplantation Findings

Spleen-brei transplants prepared from animals bearing the leukemia in the first 10 noninbred serial transplantations (generations 23–32) grew progressively in each of 57 noninbred guinea pigs that were 7 days old or less but in none of 24 animals over 48 days of age at the time of transplantation. Transplantation with brei prepared from spleens removed from noninbred animals bearing the leukemia in the next 8 transplant generations (generations 33–41) were not uniformly progressive in young animals (2 to 9 days old). In animals under 10 days of age the percentage of progressive takes from noninbred to noninbred animals dropped from 100 percent during the first 10 transplant generations to 66 percent during the next 8 generations. In animals 5 weeks of age, the percentage of progressive takes dropped from 100 percent in the first 3 noninbred serial transplantations to 11 percent in the 15th to 18th noninbred serial transplantations.

As a follow-up study, a transplant of the original line of L2B was also successfully transplanted in its *52nd* transplant generation in strain 2 guinea pigs to 2-to-4-day-old stock guinea pigs.[5] The tumor was carried in noninbred guinea pigs for 14 serial transplant generations, using 1 cc. whole spleen brei subcutaneously for the first 3 and 1 cc. of whole blood intraperitoneally for the next 11 serial transplantations. There was progressive growth of the transplant after death with leukemia in each of the 46 animals receiving the tumor homograft.

Discussion

From the present limited study in guinea pigs it is evident that with increasing age there is a decrease in the stock (noninbred) animal's ability to accept L2B as a tumor homograft. This change varies greatly with individual animals and may appear as early as the 5th day of life. It is well established in many guinea pigs after weaning age (2–3 weeks) and is completely established by the 8th week of life. During the period of study, some alterations may also have occurred in the growth properties of L2B, for after 18 serial passages in noninbred animals there was a

[5] Dr. C. A. Velat has been kind enough to permit quotation of this work, which was also performed in the Laboratory of Pathology, National Cancer Institute. Dr. Velat's present address is Providence Hospital, Detroit, Mich.

decrease in the number of progressive takes, even in young animals. A parallel study by Dr. Velat, 1 year later, showed that the original tumor grew with undiminished vigor when transferred from the inbred line to 2-to-4-day-old stock guinea pigs.[6] The results herein reported made it possible, when inbred animals became available 2 years later, for us to complete the corticosteroid study originally contemplated (*5*).

In a review of transmissible and transplantable tumors in 1953, only 2 tumors were listed as being available in the guinea pig (*12*). Both arose and were carried in inbred animals. The short supply of inbred guinea pigs may be one factor limiting cancer research in this species. Demonstration of the feasibility of transplantation from inbred to stock guinea pigs makes it possible to consider such studies in this species more practicable.

Successful homoiotransplantation in young mice and rats was known and described by early workers (*13*) as well as by more recent observers (*14, 15*). It has also been reported in young puppies (*16*). In the study reported herein, attention is drawn to the large dose of tumor cells used for transplantation and the high level of circulating endogenous cortisol (compound F) normally found in the guinea pig (*1–6*), as part of a possible combination of factors (*17*) that, together with the extreme youth of the recipients, may have been associated with a diminished immune response to the tumor homograft.

Summary

The acute lymphocytic leukemia 2B (L2B) that originated in a highly inbred strain 2 guinea pig was successfully transplanted in its 22nd transplant generation into 7-day-old noninbred guinea pigs. This lymphoma was then carried in transplant in very young noninbred guinea pigs for over 18 generations and grew progressively when transplanted back to the inbred strain of origin. Attempts to transplant the lymphoma serially to noninbred animals of different ages were more successful in animals under 10 days of age (89%) than in animals between 3 and 7 weeks of age (31%), and no takes occurred in animals over 8 weeks of age. Attention is directed to the peculiar ability of young animals to accept a tumor transplant from a genetically unrelated member of the same species.

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[6] Further evidence of the probable importance of age in homoiotransplantation was our observation that liposarcoma D4888 (originating in the strain 2 guinea pig) grew in 4-day-old but not in 45-day-old stock guinea pigs.

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PLATE 18

FIGURE 1.—Leukemic mesenteric node, *upper left;* normal node, *upper right;* leukemic spleen, *lower left;* normal spleen, *lower right.* Actual size.

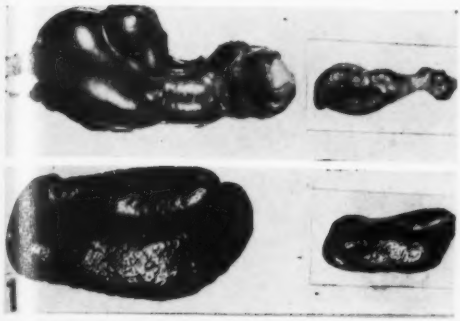
FIGURE 2.—Peripheral blood smear, leukemia 2B in noninbred Hartley guinea pig, 12 days after transplantation from inbred strain 2 donor. × 187

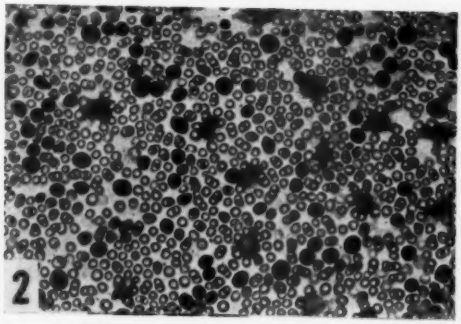
FIGURE 3.—Hemorrhagic area and surrounding leukemic infiltrations in the cerebellum of noninbred guinea pig dead with leukemia. × 65

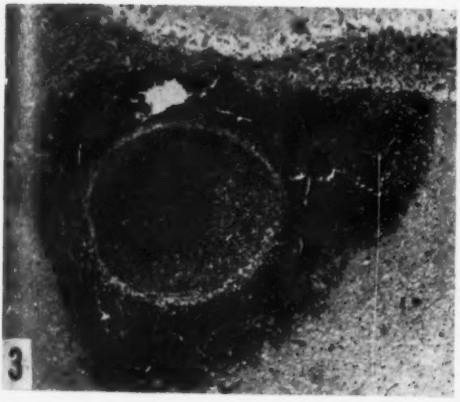
FIGURE 4.—Peripheral blood smear, leukemia 2B of figure 2 above, showing mitotic figure and 3 blast forms. × 1640

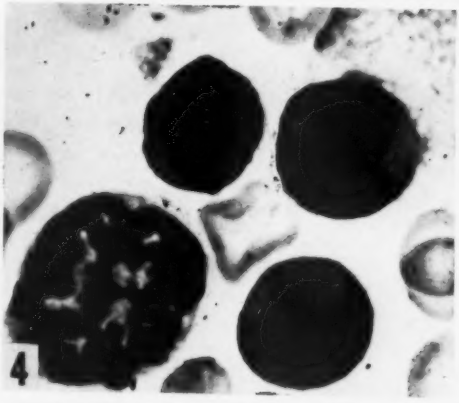
FIGURE 5.—Adrenal gland with periadrenal fat, capsule, cortex, and medulla containing leukemic infiltrations. × 65

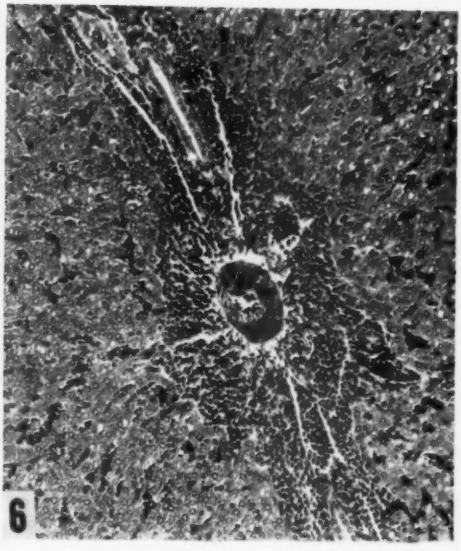
FIGURE 6.—Leukemic infiltration in liver in nodular form in periportal area. Sinusoids also prominent, with leukemic-cell aggregates. × 130











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Effect of Concentration of Benzopyrene in Skin Carcinogenesis [1]

E. L. Wynder, Lois Fritz, *and* Natalie Furth,[2]
Section of Epidemiology, Division of Preventive Medicine, Sloan-Kettering Institute, New York, New York

The concept that the concentration of an injected carcinogen has a direct relation to the incidence and the latent period of subcutaneously induced tumors has been well established (*1–3*). Basic data on the quantitative aspects of tumor formation with benzopyrene, through skin application, have been presented by Horton and Denman and by Poel and Kammer (*4, 5*). Because of possible variations in painting technique and solvents used, it seemed pertinent to determine the activity of varied benzopyrene concentrations and to establish the minimum dose of benzopyrene capable of producing skin cancers in mice and rabbits with the same technique of application employed in previous studies (*6–8*). The present study was also undertaken to indicate whether the benzopyrene content of cigarette-smoke tar alone can account for the positive animal results.

Methods

The experiment was conducted with 2 strains of mice, Swiss (Millerton Farms, Millerton, N. Y.) and C57BL (Lieberman Farms, New London, Conn.), and 1 hybrid, CA F_1 (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.), and with rabbits of the New Zealand strain. Each experimental group consisted of 20 mice and 3 rabbits.

Benzo[*a*]pyrene (Eastman) was diluted with acetone (gm./100 ml.) in concentrations varying from 0.5 to 0.0001 percent. The solutions were freshly prepared every 2 weeks and applied with a #5 camel's hair brush to the entire backs of mice 3 times a week and to a 5×5 cm. area of the backs and the entire ears of rabbits 5 times a week. The backs of all animals were shaved whenever hair growth recurred. The benzopyrene-acetone solutions were stored in clear glass bottles in a refrigerator at 7° C. A separate shaving head was used with each concentration of he benzopyrene solution. In using the term "dose," we are aware that it cannot be applied as accurately in a painting experiment as in one in which the material is injected into a given animal. More pertinent than

[1] Received for publication April 3, 1957.
[2] We are grateful for the assistance of Dr. I. Bross and Mrs. R. Feldman in connection with the statistical analysis of this data.

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the results of any single experimental group is the comparison of the different groups.

Results

Mice

Tumor formation.—The percentages of papilloma and cancer produced in the 2 strains of mice and the hybrids for each concentration of benzopyrene solution are shown in tables 1 to 3. The time of first appearance of both papillomas and cancers is based upon gross observation. Some of the cancers arose from ulcers rather than papillomas.

The percentages of papilloma and cancer formation were similar for all 3 strains of mice. The differences found are not significant, particularly if one considers that the survival time among the CA F_1 mice is longer than among the other 2 strains. The 0.001 percent solution produced some tumors among CA F_1 mice; the first papilloma appeared at 13 months and the first cancer at 16 months. No lesions were observed among Swiss or C57BL mice. The latter, however, had a high mortality, with none of the mice surviving 11 months. Thus, one cannot compare the susceptibility of C57BL and CA F_1 mice to this concentration. With the 0.0005 percent solution 1 single papilloma was noted among the C57BL mice in the 18th month. No lesions were observed with the 0.0001 percent solution.

A primary difficulty with the analysis of this data is that there is a competition of risks—the animals can die from toxic effects of the agent as well as from cancer. In order to deal with the competition problem a device devised by Bryan and Shimkin was employed (*1*). However, this is a simplified method for dealing with what is actually an extremely involved problem. As will be seen from the data, the toxic effect is still visible in the data after the adjustment has been made (text-fig. 1). All 3 of the quantal response curves show a downward turn at the top benzopyrene dose. This downturn probably is due entirely to the toxic effect.

At this stage it is convenient to think in terms of the TD50, the dose of the agent that will produce tumors in 50 percent of the mice within the time span of this experiment. We are really more interested in the "threshold," which might be taken as the TD 0.5, but to estimate this quantity accurately would require larger groups of mice.

Both strains of mice and the hybrids exhibit quantal response curves that are quite similar in certain essential features. The adjusted response at 0.005 percent benzopyrene solution is greater than 75 percent for all 3. The response to 0.001 is less than 25 percent. Hence, the TD50 is between 0.001 and 0.005 in all 3 cases. If there were no deaths except from cancer in a series of 20 mice and if the dose given was the TD50, then we would expect to find that between 25 and 75 percent of the mice would develop tumors, that is, 95 percent of the time there would be more than 5 animals and less than 15 animals that would develop tumors. This expectation is the basis of the assertion that the TD50 is between 0.001 and 0.005. The deaths due to toxic effects may introduce some error into this calculation.

TABLE 1.—*Number of papillomas and cancers by month—Swiss mice*

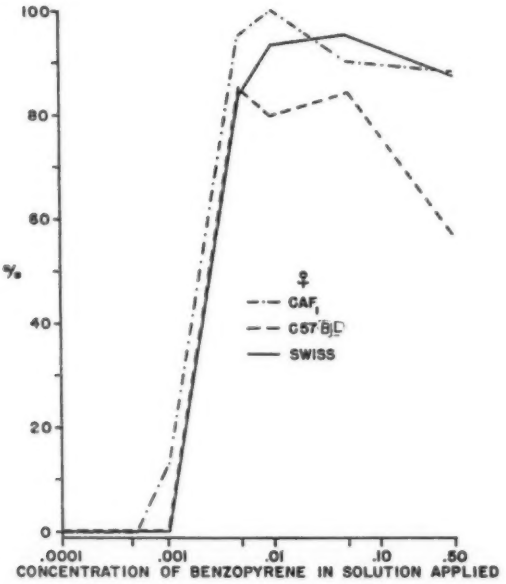
No. months	Percentages of benzopyrene in acetone:																				
	0.5			0.05			0.01			0.005			0.001			0.0005			0.0001		
	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C
1	20	0	0	20	0	0	20	0	0	20	0	0	20	—	—	20	—	—	20	—	—
2	19	8	0	20	0	0	20	0	0	19	0	0	20	—	—	20	—	—	20	—	—
3	17	14	4	20	2	0	20	1	0	19	0	0	20	—	—	20	—	—	19	—	—
4	17	14	4	20	13	2	20	3	0	19	0	0	20	—	—	20	—	—	19	—	—
5	8	14	12	13	13	7	20	3	0	19	0	0	20	—	—	20	—	—	19	—	—
6	0	15	15	0	16	19	19	3	0	19	0	0	19	—	—	20	—	—	19	—	—
7							19	9	3	16	2	0	19	—	—	20	—	—	18	—	—
8							11	17	8	15	3	1	19	—	—	17	—	—	18	—	—
9							8	17	10	13	3	1	18	—	—	17	—	—	18	—	—
10							8	17	13	11	4	2	16	—	—	17	—	—	18	—	—
11							1	17	16	10	7	3	16	—	—	14	—	—	18	—	—
12							0	17	17	9	8	5	15	—	—	14	—	—	16	—	—
13										3	9	9	13	—	—	14	—	—	15	—	—
14										2	11	10	11	—	—	12	—	—	13	—	—
15										2	11	11	7	—	—	12	—	—	11	—	—
16										1	11	11	7	—	—	11	—	—	9	—	—
17										1	11	11	6	—	—	9	—	—	7	—	—
18										0	11	11	6	—	—	7	—	—	4	—	—
19													4	—	—	4	—	—	1	—	—
20													0	—	—	2	—	—	0	—	—
21																0	—	—			

TABLE 2.—*Number of papillomas and cancers by month—CA F_1 mice*

No. months	Percentages of benzopyrene in acetone:																				
	0.5			0.05			0.01			0.005			0.001			0.0005			0.0001		
	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C
1	20	0	0	20	0	0	20	0	0	20	0	0	20	0	0	20	—	—	20	—	—
2	20	7	0	20	0	0	20	0	0	20	0	0	20	0	0	20	—	—	20	—	—
3	20	11	0	18	2	0	20	0	0	20	0	0	20	0	0	20	—	—	20	—	—
4	18	20	6	17	14	0	20	0	0	20	0	0	20	0	0	20	—	—	20	—	—
5	6	20	13	14	17	3	20	1	0	20	0	0	20	0	0	20	—	—	20	—	—
6	0	20	17	3	17	12	20	1	0	20	1	0	20	0	0	20	—	—	20	—	—
7				0	17	15	19	1	0	19	1	0	20	0	0	20	—	—	20	—	—
8							18	5	2	19	1	0	19	0	0	20	—	—	20	—	—
9							16	9	3	19	2	0	19	0	0	20	—	—	20	—	—
10							13	15	8	19	3	0	19	0	0	20	—	—	20	—	—
11							5	17	15	19	4	0	19	0	0	20	—	—	19	—	—
12							2	18	18	17	9	3	19	0	0	19	—	—	19	—	—
13							0	18	19	10	14	7	19	1	0	18	—	—	19	—	—
14										7	14	10	19	2	0	17	—	—	19	—	—
15										7	17	12	19	2	0	16	—	—	18	—	—
16										3	18	15	17	4	1	15	—	—	17	—	—
17										2	19	17	14	4	1	15	—	—	14	—	—
18										1	19	17	13	4	1	14	—	—	13	—	—
19										0	19	17	11	5	1	14	—	—	10	—	—
20													8	6	2	11	—	—	6	—	—
21													7	6	2	9	—	—	3	—	—
22													5	6	2	5	—	—	2	—	—
23													3	6	2	3	—	—	2	—	—
24													0	6	2	0	—	—	0	—	—

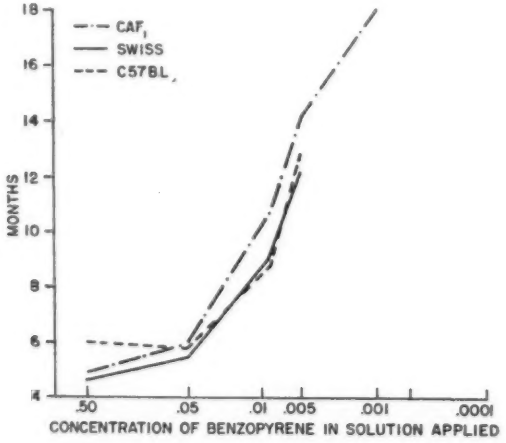
TABLE 3.—*Number of papillomas and cancers by month—C57BL mice*

No. months	Percentages of benzopyrene in acetone:																				
	0.5			0.05			0.01			0.005			0.001			0.0005			0.0001		
	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C	No. survivors	P	C
1	20	0	0	20	0	0	20	0	0	20	0	0	20	—	—	20	0	—	20	—	—
2	20	0	0	20	0	0	20	0	0	20	0	0	20	—	—	20	0	—	20	—	—
3	20	2	0	19	0	0	20	0	0	20	0	0	20	—	—	20	0	—	20	—	—
4	19	14	0	14	5	1	20	0	0	20	0	0	20	—	—	20	0	—	19	—	—
5	10	16	4	8	14	6	20	0	0	20	0	0	19	—	—	18	0	—	18	—	—
6	4	16	4	7	14	6	20	4	0	20	0	0	14	—	—	18	0	—	16	—	—
7	0	16	8	0	14	12	19	11	3	19	1	1	11	—	—	15	0	—	15	—	—
8							13	16	6	19	2	1	8	—	—	13	0	—	9	—	—
9							12	16	9	19	3	1	5	—	—	12	0	—	7	—	—
10							3	16	14	18	5	2	2	—	—	12	0	—	5	—	—
11							0	16	15	17	8	3	0	—	—	12	0	—	5	—	—
12										14	15	4				10	0	—	2	—	—
13										7	19	9				9	0	—	2	—	—
14										2	19	13				8	0	—	2	—	—
15										2	19	15				7	0	—	2	—	—
16										0	19	15				7	0	—	0	—	—
17																4	0	—			
18																4	1	—			
19																4	1	—			
20																3	1	—			
21																0	1	—			



TEXT-FIGURE 1.—Adjusted percentages of mice developing cancer.

A decrease in dose is followed not only by a decrease in the percentage of animals developing cancer but also by a lengthening of the time between the initial painting and the appearance of cancer (text-fig. 2).



TEXT-FIGURE 2.—Average duration to death for animals dying of cancer—benzopyrene series.

TABLE 4.—*Number of papillomas and cancers by month—rabbits*

No. months	Percentages of benzopyrene in acetone:																
	0.5						0.005				0.01			0.005	0.001	0.0005	0.0001
	Survivors			No. tumors on back		No. tumors on left ear	Survivors		No. P on back	No. P on left ear	Survivors		No. P on back	No. P on left ear	No. survivors	No. survivors	No. survivors	No. survivors
	No.	No. with P	No. with C	P	C	P	C	No.	No. with P			No.	No. with P					
1	3							3				3				3	3	3	3
2	3	1		1		1		3				3				3	3	3	3
3	3	2		6		2		3				3				3	3	3	3
4	3	3		15		3		3	1	2		3				3	3	3	3
5	3	3		21		6		3	1	2		3				3	3	3	3
6	3	3		24		9		3	2	3	1	3				3	3	3	3
7	3	3		24		9		3	2	3	1	3				1	2	3	2
8	3	3		39		11		3	2	4	1	3				1	2	3	2
9	3	3		49		12		3	2	4	1	3				1	2	3	2
10	3	3		50+		17		3	2	4	4	3				1	2	3	2
11	2	3	1	50+		50+	1	2	3	8	5	3	1	1		1	2	3	2
12	2	3	1	50+	1	50+	1	2	3	14	6	3	1	1		1	2	3	2
13	1	3	2	50+	1	50+	1	2	3	17	6	3	2	2	1	1	2	3	2
14	1	3	2	50+	1	50+	1	2	3	31	7	3	2	3	1	1	1	2	2
15	1	3	2	50+	1	50+	1	2	3	37	13	3	2	3	1	1	1	2	2
16	1	3	2	50+	1	50+	1	2	3	37	15	2	2	3	1	1	1	2	2
17	1	3	2	50+	1	50+	1	2	3	37	15	2	2	3	1	1	1	2	2
18	1	3	3	50+	2	50+	2	2	3	50+	50+	1	2	3	1	1	0	2	2
19	0	3	3	50+	2	50+	2	2	3	50+	50+	1	2	3	1	1		2	2
20								2	3	50+	50+	1	2	3	1	1		2	2
21								2	3	50+	50+	1	2	3	1	1		2	2
22								2	3	50+	50+	1	2	3	1	1		2	2
23								2	3	50+	50+					1		2	2
24								2	3	50+	50+							2	2

Rabbits

Rabbit skin is far more resistant to benzopyrene than was demonstrated for the mice (*9*) (table 4). Cancers were produced only with a 0.5 percent solution—the first cancer appearing at 11 months. All 3 rabbits had cancer by the 18th month. We compared the number of lesions that appeared on the back, where a 5 × 5 cm. area was painted, with those that appeared on the ear. Papillomas tended to occur somewhat earlier on the back than upon the ear. Only occasional papillomas were produced with 0.01 percent and none with the lower dilutions.

Discussion

Much emphasis has been placed, especially by Kennaway and by Poel, on the possibility that prolonged application with a weak carcinogen can produce a significant number of tumors (*10*, *11*). Poel and Kammer have shown that with the drop method cancers can be obtained with as little as 0.01 percent per application (*5*). In previous studies on skin painting, the lowest concentration of benzopyrene that produced tumors was 0.02 percent (*12*). The lowest amount of benzopyrene with which subcutaneous tumors in mice have been produced is 0.0004 mg. (*13*).

The lowest concentration, in this study, that produced papillomas in mice was 0.0005 percent, which, however, yielded only a single papilloma. The lowest concentration which produced cancer was 0.001 percent. These experiments have borne out Kennaway's and Poel's contentions, though there is obviously a level below which even lifelong exposure to a given amount of carcinogen will not produce tumors in the experimental animal.

Rabbits, as shown in previous studies by Berenblum, show greater resistance to benzopyrene than mice (*9*). In fact, cancers could be obtained only with the 0.5 percent solution after 2 years of application. It is conceivable that had we prolonged the application of the weaker solutions of benzopyrene cancers might have developed, though the low papilloma yield does not suggest this.

The susceptibility of the mouse strains and hybrids to benzopyrene did not show any consistent variation, though the CA F_1 mice had more tumors with the 0.001 percent concentration. Such a finding may be coincidental, particularly in view of the fact that, in general, the latent period was slightly shorter among Swiss and C57BL mice. The CA F_1 mice had a longer survival rate than Swiss and C57BL mice in this experiment. These results are at some variance with the tobacco studies in which Swiss mice were found to be significantly more susceptible to tumor formation than CA F_1 and C57BL mice (*7*).

The general principles of latent period and number of tumors formed as they relate to dose are too well known to be reviewed here. The data as shown are consistent with the established views that the potency of a carcinogen has a direct bearing on the number of tumors and latent period for appearance of tumors in the experimental animal.

In the present studies it is realized that the term "dose" does not have the same meaning as it has in an experiment in which one injects a known

amount of carcinogen into an animal or applies an exact amount of carcinogen to the skin. In the skin-painting technique, as used here, one can obtain only an approximate idea about the amount of carcinogen given to each animal. However, this type of application of carcinogen is akin to that in human exposure and more important than the evaluation of a given single experimental group is the comparison from group to group. From this point of view, it may be pertinent to determine how the results of this particular investigation pertain to some of the recent data established in the field of tobacco carcinogenesis.

Relation to tobacco carcinogenesis.—Because of the particular emphasis placed upon benzopyrene as a factor in tobacco carcinogenesis, it appeared important to establish a base line for the activity of benzopyrene on mouse and rabbit skin, with the same solvent and skin-painting techniques as in our tobacco-tar studies. Benzopyrene constitutes not more than 2 p.p.m. of smoked tobacco tar (*14*). A 50 percent cigarette tar-acetone solution is estimated to have a carcinogenic activity equal to that of about 0.004 percent benzopyrene solution in acetone for mice and about 0.05 percent for rabbits (*14*). The activity of the fraction eluted with hexane from neutral cigarette-tar fraction in a 10 percent concentration is about 0.05 percent in terms of benzopyrene for rabbits. The activity, for mice, of the fraction eluted with carbon tetrachloride from the neutral cigarette fraction in a 10 percent concentration is about 0.005 percent in terms of benzopyrene. Neither of these 2 eluates contains benzopyrene in excess of 2 p.p.m. These data, therefore, again demonstrate that benzopyrene cannot be the only, and most likely not the major, carcinogen in cigarette tar. It is of interest that both these fractions present spectrographic evidence of higher aromatic hydrocarbons in 50 times the concentration of benzopyrene. The present data thus show that neither the results in mice nor, more notably, in rabbits on tobacco carcinogenesis can be the sole consequence of the benzopyrene content of tobacco tar. This does not deny the possibility that a cocarcinogen present in tobacco tar might potentiate the benzopyrene activity present in cigarette tar or that we may have a summation effect of several aromatic polycyclics (*15*, *16*).

From the practical point of view, the most striking feature of the present study is the fact that a fivefold dilution of a 0.005 percent benzopyrene solution in acetone changes the response from one where nearly all the animals develop cancer to one where very few develop cancer. The environmental studies suggest that if a corresponding dose-response curve were constructed for humans in relation to tobacco smoking, one would find that even if the exposure to tobacco tar were only cut in half by effective filtration or modification of the tobacco, there might be a substantial reduction in the number of smokers who develop cancer of the respiratory tract.

Summary and Conclusions

1) With the skin-painting technique, 2 strains of mice, 1 hybrid, and rabbits have been painted with different concentrations of benzopyrene-acetone solutions.

2) No significant differences in tumor susceptibility were found between CA F_1, Swiss, and C57BL mice.

3) Mouse epithelium was significantly more sensitive to tumor formation after application of benzopyrene than was rabbit epithelium.

4) These studies show that the benzopyrene content of cigarette tar, which does not exceed 2 p.p.m., is not sufficient to account solely for the established carcinogenic activity of cigarette tar in mice and rabbits.

5) Emphasis has been placed on the importance of determining dose-response levels of carcinogens and on their relation to practical preventive measures.

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Plasma-Cell Neoplasms Beginning in the Ileocecal Area in Strain C3H Mice [1]

THELMA B. DUNN, *Laboratory of Pathology, National Cancer Institute,* [2] *Bethesda, Maryland*

Plasma-cell neoplasms in mice have been described by Rask-Nielsen and Gormsen, who observed spontaneous and induced plasma-cell leukemias first in strain Street mice (*1*) and later in various other strains (*2*), and by Bichel (*3*), who described a plasma-cell leukemia in strain AK mice.

In the strain Street mice most of the plasma-cell neoplasms were classified as leukemia, since the cells had the same distribution as the neoplastic cells in stem-cell leukemia. Only 5 neoplasms were considered to be localized plasmacytomas and these were found in mice 14 or more months of age. Injection of carcinogenic hydrocarbons did not accelerate the development of plasma-cell leukemia. The authors considered it probable, however, that 4 of the 5 local plasmacytomas were induced by the carcinogen, since they occurred at the site of injection. The neoplastic cells differed widely in degree of differentiation. The cells of some of the neoplasms closely resembled normal plasma cells, while those of other neoplasms were so undifferentiated that they resembled neoplastic reticulum cells. Plasma-cell neoplasms were later described by these authors in various other strains of mice (*2*). Twenty-six cases were considered to be spontaneous and 24 induced. The induced neoplasms occurred at an earlier age, but were similar in all other respects to the spontaneous tumors. One relatively undifferentiated tumor was successfully transplanted. No abnormal serum proteins were found in the hosts.

Bichel (*3*) described a plasma-cell neoplasm that was readily transplanted in AK mice. No serum protein analysis of mice bearing the tumor was reported. He also reported that cells from a plasma-cell neoplasm grew in combination with chicken fibroblasts for 8 months in tissue culture (*4*).

The plasma-cell neoplasms included in this report have all been found in strain C3H mice, or its hybrids, except for 1 in a Carworth Farms #1 mouse. All originated in a localized area of the gastrointestinal tract at the ileocecal junction, except for 1 tumor which was found at the gastroduodenal junction. A wide range in the amount of spreading and in the

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degree of differentiation of the neoplastic cells was noted. Two of the neoplasms have been transplanted successfully in this laboratory and 1 has been converted to the ascites form (*5*). The present paper deals chiefly with the incidence and morphologic features of the primary tumors and gives some information on the morphology and behavior of the transplanted tumors when carried as solid-tumor lines.

Generalized plasma-cell neoplasms, such as were described by Rask-Nielsen and Gormsen, have also been observed in our strain C3H mice, but these are not considered in the present report.

Primary Neoplasms

Groups of mice in which neoplasms developed.—Although plasma-cell neoplasms are rare, it is probable that many more occur than are recognized, since a careful search of the ileocecal area is not routinely made at autopsy and tissues from this area are not regularly taken from all old mice of strain C3H for histologic study. The lesion in the ileocecal area is easily overlooked. When small, it may be mistaken for an area of inflammation with ulceration. When larger, the mesenteric node becomes involved and may be so large that it overshadows the lesion in the intestine. In this laboratory many of the neoplasms have been found in mice in which the ileocecal area was examined with care. Although the tumors were found in 2 groups of mice that had received carcinogenic agents, they were also found in untreated mice from many different groups, and the part played by carcinogenic agents is debatable.

Neoplasms composed of plasma cells and originating in the ileocecal region have been found in the following groups of mice:

1) *Mice given a variety of substances by stomach tube in an attempt to induce gastric cancer* (*6*). At autopsy, the entire gastrointestinal tract was examined under magnification after it had been opened, following the technique described by Stewart and Lorenz (*7*). Of 31 strain C3H mice that received an emulsion of dibenz[*a*,*h*]anthracene, 6 developed a lesion at the ileocecal junction. The lesion was thought at autopsy to be an inflammatory reaction, and no attempt was made to transplant it (fig. 1).

2) *Mice of strain C3H/He, injected subcutaneously with 3-methylcholanthrene (MCA) when 1 to 2 weeks, 3 months, or 1 year of age.* The mice were then killed whenever subcutaneous nodules were palpated (*8*). Thirty mice remained when the experiment was terminated and these were kept for observation. One male mouse that received MCA when 15 days old had an enlarged abdomen at the age of 14 months. At autopsy, the intestinal wall in the ileocecal area was thickened and the mesenteric node was enlarged. Tissue from the node was transplanted successfully, and the tumor line was designated 70429. Details about this tumor are described in a later section. Of the remaining mice, two, 26 and 28 months old, were found to have ulceration of the mucosa at the ileocecal junction and enlargement of the mesenteric node. On microscopic examination, there was infiltration of the gut wall with plasma cells.

Tissue from the intestinal lesions in these mice did not grow when inoculated subcutaneously.

3) *Mice of strain C3H, 7 of which received injections of stilbamidine to see whether any effect was produced on plasma cells and 10 of which were used as controls in this experiment.* At 13 months of age, an untreated control mouse developed a plasma-cell lesion in the ileocecal area. No effect of stilbamidine was noted on plasma cells in the experimental mice.

4) *Mice of strain C3H kept as stock mice or used as controls in a variety of experiments at the National Cancer Institute.* No record was made of the total number examined, but several mice from these groups have had plasma-cell tumors. It can be roughly estimated that 1 in every 100 old C3H mice developed a plasma-cell tumor, but as stated, the number has varied with the thoroughness of the autopsy. In several mice, only the mesenteric node containing neoplastic plasma cells was taken at autopsy, but it is probable that the ileocecal area was the primary site.

5) *Mice from other laboratories.* Plasma-cell neoplasms have been found several times in histologic sections of unusual neoplasms submitted to this laboratory for an opinion. One was found in a Carworth Farms #1 female mouse, 24 months old, from the laboratory of Dr. M. P. Finkel at the Argonne National Laboratory. This neoplasm is remarkable because it is the only one found in a strain of mouse other than strain C3H or its hybrids. Several plasma-cell tumors have been seen in sections submitted from the Cancer Research Genetics Laboratory, Berkeley, California, by Dr. H. I. Pilgrim. He transplanted 2 of these neoplasms successfully and sent us mice bearing the second transplant generation of 1 of them. This tumor arose in a 22½-month-old female C3H/He mouse that had been gonadectomized at 2 months of age. The primary lesion and the transplant line (X5563) are described in a later section.

A plasma-cell tumor found at the junction of the stomach and duodenum by Miss Delta Uphoff, of the Laboratory of Biology, National Cancer Institute, resembled the ileocecal tumors. The mouse was from strain C3Hf/He and at 3 months of age received 800 r irradiation followed by an injection of bone marrow from a BALB/c mouse. When the animal was 1 year old it was killed and an autopsy performed. The histology of the tumor found at the gastroduodenal junction is described in a later section.

Morphology

Primary neoplasms.—The smallest plasma-cell lesions recognized grossly consisted of an area less than ½ cm. in diameter in which the wall of the intestine in the ileocecal area was thickened and hyperemic. The muscosa overlying the lesion was ulcerated. Histologically, the muscle coats of the intestine were infiltrated with well-differentiated plasma cells (figs. 1, 2, 3); similar cells could often be found in adjacent lymphatic channels in the mesentery and occasionally in the sinuses of the mesenteric lymph node.

The 2 plasma-cell tumors that grew when transplanted to other mice showed more widespread involvement of the tissues of the donor animal.

In 1 of these tumors, 70429, which originated in a mouse in this laboratory, the intestinal neoplasm measured 1 cm. in length and the ulcer in the overlying mucosa measured 0.5 cm. (fig. 4). Nodular masses of soft, grayish-white tissue were found in the adjacent mesentery and in the lower end of the mesenteric node, which was soft, white, and enlarged. Both the spleen and thymus were enlarged but the other lymph nodes were not. Histologically, the plasma cells in the intestinal wall showed great variation in the degree of differentiation. Neoplastic cells in the mesenteric node and thymus were less differentiated than many cells in the ileocecal area. Neoplastic cells replaced the centers of the splenic nodules and a few small collections of neoplastic plasma cells were found in the liver.

The second plasma-cell tumor that grew on transplantation, X5563, was sent to us by Dr. H. I. Pilgrim. The mouse in which the tumor arose showed nodules of neoplastic tissue surrounding the gut in the ileocecal region, and the mesenteric node was enlarged. Histologically, the neoplastic cells of this tumor were well differentiated, resembling normal plasma cells. They infiltrated the wall of the lower ileum (fig. 5) and cecum (fig. 6) and were present in the mesenteric node.

The small local lesions and the 2 neoplasms successfully transplanted represent the extremes in size and extent of involvement by these plasma-cell tumors. The other tumors observed fell between these extremes and ranged from small growths localized in the ileocecal area to large tumors involving the mesenteric node.

The primary tumor found at the gastroduodenal junction was like the ileocecal tumors in all respects except location. The neoplastic cells had spread beyond the gastric wall and had invaded the neighboring gastric node (fig. 7). Microscopic sections of the lymph node allowed for a close comparison between the normal plasma cells in the medulla of the node and neoplastic cells in an adjacent mass of neoplastic tissue. The 2 types of cells were separated at one point by only a narrow lymphatic sinus (fig. 8). As compared with the normal plasma cell the neoplastic cell was larger and the nuclei were more hyperchromatic, but the cells still retained characteristics of the plasma cell, such as an eccentric nucleus with chromatin clumping and nongranular basophilic cytoplasm with a central clear area next to the nucleus.

Biologic Behavior and Morphology of the Transplanted Tumors

Tumor 70429 was very erratic during early generations of its transplant history following subcutaneous inoculation of tumor tissue by the trocar method. For the first 5 generations, progressive growth occurred in only 47 of 152 mice inoculated. The time when the tumor became palpable ranged from 25 to 172 days. At the time of autopsy, only local subcutaneous masses were found in 29 of the 47 mice, the peripheral lymph nodes only were involved in 3, and the gonads only in another 5. In the remaining 20 mice, there was involvement of several sites or widespread dissemination. The tumor grew in 20 male mice, and in 3 of

these the testes were infiltrated by tumor cells. Among the 27 female mice, the ovaries were involved in 10. At autopsy, the growth in the ovaries was often massive (fig. 9). In later generations, neoplastic cells could often be found in the ovaries on microscopic examination (fig. 10), but gross enlargement of the ovaries no longer developed.

Attempts were made to modify the transplantation behavior of this tumor. Intraperitoneal transplantation was no more successful than the subcutaneous route, and the tumor grew no more readily in irradiated, newborn, or in cortisonized mice. After 4 years of continual subcutaneous transplantation, during which 34 transfers have been made by selecting tissue from rapidly growing local tumors, the neoplastic line is fairly stable and growth at the site of inoculation becomes palpable in about 3 weeks in nearly all mice. At the present time the internal organs usually contain only microscopic accumulations of tumor cells, while the local tumor attains a large size.

Microscopically, the tumor now appears much as it did originally (fig. 11a). The cells are larger and the cell margin is less distinct than in the normal plasma cell. The nucleus is relatively large and the characteristic chromatin clumping of the plasma cell cannot be seen. Only occasional cells show a clear central area. Mitotic figures are frequent. Besides the neoplastic cells, large cells with faintly eosinophilic cytoplasm and a single pale nucleus are always present. Small particles resembling cellular debris are often found in these cells. In the ascites form, analogous pale "foam" cells with an indistinct, faintly stained nucleus and basophilic particles in the cytoplasm were found evenly distributed among the neoplastic cells (fig. 11b). The morphology and the particles within the cytoplasm suggest that these cells are macrophages, but the regular distribution among the tumor cells, often in areas free of necrosis, and the peculiar morphology of these cells in the ascites form are different from the ordinary macrophage infiltration in necrotic areas of neoplasms. For these reasons, the possibility cannot be dismissed that they are modified tumor cells, although no transitional forms have been observed. A remarkable feature in some tumors in the early generations was neoplastic cells containing a substance that suggested a Russell-body type of formation (fig. 12). The cells contained varied amounts of an eosinophilic or fuchsinophilic substance, sometimes in such quantity as to disrupt the cell membrane and appear extracellularly. The substance was less eosinophilic and less intensely fuchsinophilic than the Russell bodies in non-neoplastic cells, and it was in an amorphous state rather than in intracellular globules. The nuclei in the modified cells were small and pyknotic, as in cells with Russell bodies. This modification has not appeared in recent generations.

The ascitic form of neoplasm 70429 was developed by Potter from the 20th transplant generation of the solid line. He has described its mode of infiltration and the length of life of mice bearing transplants of this neoplasm (*5*). The ascitic cells have been uniform throughout their history. Under the phase microscope, they reveal a very characteristic

pattern. There is a dense nuclear membrane with ill-defined dense areas in the nucleus. The cytoplasm is relatively scant and the cells often show "bubbling" of the cell membrane. This picture is also seen with Wright's stained smears, and the nuclear membrane and the dense chromatin areas are characteristic (fig. 11b). This neoplasm has been found to be very sensitive to azaserine and 6-diazo-5-oxo-L-norleucine (DON) (*5*). It has not altered the plasma proteins, and Bence Jones protein has not been found in the urine.

Among the many mice bearing plasma-cell tumor 70429 that were examined, 3 had an unusual renal lesion that has not been recognized in non-tumor-bearing mice. Multinucleated syncytial masses have been found within the proximal tubules, most often near the medulla (fig. 13). Some of these syncytial masses appeared to be fused tubular epithelial cells that were sloughed off and formed a multinucleated cellular cast within the lumen. These casts were sometimes surrounded by tubular epithelial cells still attached to the basement membrane (fig. 14). One of the mice with this kidney lesion had received azaserine but the others had not been treated. Special and unusual conditions must be required for producing these infrequent renal lesions. Casts of amorphous material, such as are found in the tubules of human "myeloma kidneys," have not been a prominent feature, although an indistinct eosinophilic material may appear within the multinucleated syncytial masses. No calcification has been noted.

After transplantation, tumor X5563 grew in about two thirds of strain C3H/He mice inoculated in this laboratory. Growths were palpable in 2 or 3 weeks and the tumor is now routinely transplanted every 60 days. At autopsy, the local subcutaneous tumors usually appeared congested and were a purplish color. The tissue was moderately firm, yet easily squashed under a coverslip, so that individual cells could be separated for observation. An unusual feature at autopsy was a 1 to 3 mm. zone of edematous tissue surrounding most of the tumors. The lymph nodes in the tumor-bearing mice were smaller than normal.

Microscopically, the neoplastic cells of the first transplant generation had a close resemblance to normal plasma cells, the only striking difference being the considerable increase in size and frequent binucleate cells and mitotic figures. The nucleus was eccentric, and many cells had a distinct central clear area (fig. 15). This tumor has not been converted to an ascites form, but after intraperitoneal transplantation many of the cells are free and can be recovered in peritoneal fluid. The morphology of these detached cells has been described (*9*).

When the solid tumors were allowed to grow for a period of 3 months, neoplastic cells sometimes spread to the bone marrow and caused destructive lesions in the bone. These lesions did not contain osteoclastic or osteoblastic cells. The osseous tissue seemed to disappear abruptly as in osteolytic lesions, leaving only a fibrous covering of the marrow space (fig. 16). Neoplastic cells often extended through the openings and infiltrated the adjacent tissue. Punched-out areas were easily visualized in bones freed of all soft tissue by papain (*9*).

After 11 months the solid-tumor line is in the fourth transplant generation. The tumors become palpable in about 1 month and kill the mice in 3 to 4 months. Besides the lesion in the bone, mice bearing this plasma-cell tumor often show changes in the lymph nodes, characterized by a reduced number of lymphocytes, absence of germinal-center activity, and an increase in reticuloendothelial cells in the sinuses.

The incidence of amyloidosis has not been increased appreciably in mice bearing tumors of either transplanted line, and no Bence Jones protein has been demonstrated in the urine. The tumor tissue itself has not shown amyloid deposition.

Discussion

These plasma-cell neoplasms in the mouse illustrate many of the general characteristics of neoplastic growth. They are also analogous in many respects to multiple myeloma in man and warrant some comparison with this disease. They offer certain advantages for study because they are composed of a single distinctive cell type, and the neoplastic change can be observed within an isolated cell uncomplicated by the organoid structure of many glandular tumors.

The importance of genetic constitution is illustrated by the almost exclusive occurrence of this tumor in a single inbred strain of mouse. The plasma-cell neoplasms described in other inbred strains have not originated in a single restricted area.

Our uncertainty as to how much influence carcinogenic agents have in the genesis of some neoplasms is applicable to these tumors. Many of them arose in untreated mice and others arose in mice subjected to a variety of different procedures, such as exposure to chemical carcinogens, irradiation, and gonadectomy. The low natural incidence and the advanced age of the animals in which the tumors were found indicate that the tendency toward development of plasma-cell tumors is slight, although small lesions are probably overlooked often. These conditions make it hard to gauge the effect of chemical carcinogens, but it seems probable that they were a factor, since some neoplasms in treated mice were found at an unusually early age. This question can be settled only after a study of large groups of strain C3H mice under carefully controlled conditions.

The origin of the plasma-cell tumor at a particular anatomical site and the evidence of a preneoplastic, possibly inflammatory, lesion at this site are noteworthy. The small lesions were at first suspected to be inflammatory, and their localization at a preferential site for regional ileitis in man and cecitis in the rat (*10*) was noted. The plasma cell is widely distributed in mice, and some plasma cells are usually found in lymphatic and areolar tissues. The plasma cells in the ileocecal area are presumably the same as those found elsewhere in the body. Therefore, the neoplastic change in plasma cells in the ileocecal area suggests that the cells located here are exposed to some environmental condition, possibly a chemical carcinogen, that favors the neoplastic change. If the ulceration is primarily inflammatory and precedes the neoplastic change, the break in the

mucosa might allow a carcinogenic agent to pass through and make contact with susceptible plasma cells. Plasmomas are reported in the conjunctiva in human beings affected with trachoma (*11*) but the etiologic relationship is debatable. We are still uncertain of the relationship between inflammation and cancer—the inflammatory process may do no more than permit contact with a carcinogen. However, the plasma-cell lesion may be neoplastic from the beginning, and the failure to transplant small tumors may occur because tumor progression has not gone far enough to permit independent growth when transplanted. Other examples of selective localization of cancer in a widely distributed system of cells can be cited. Lymphocytic neoplasms regularly begin in the thymus in some inbred strains of mice (*12*), and reticulum-cell sarcomas are often found beginning in the uterine or vaginal wall in female mice (*13*). In man, the intestinal wall is often the site of lymphosarcomas, and Hodgkin's paragranuloma is reported most often in cervical nodes (*14*).

The close association of what appears to be a non-neoplastic phagocytic cell with the neoplastic plasma cells in 1 transplant line has parallels in other tumors. Normal mast cells have been described in close association with different types of neoplasms in animals and man (*13*). A large macrophage has been noted in chloroleukemia in mice (*15*) and in rats (*13*). In man, eosinophils are often associated with Hodgkin's disease. The way in which neoplastic cells attract, or "carry," normal cells has not been explained.

The transplantation behavior of one of the plasma-cell tumors illustrated the degrees by which transplanted tumor lines may become adapted and finally grow readily in all mice of an inbred strain. Originally, line 70429 grew only in about one third of strain C3H mice, but it can now be transplanted readily and it kills the host mice in a shorter and more predictable period of time than it did in early generations. This tumor also illustrated how characteristics may appear and disappear in transplanted tumors. The Russell-body modification and localization of growth in the ovary were found for a few generations and then failed to appear. This appearance and disappearance of a character in a transplant line was described by Murray in an early report on the squamous-cell modification in a mammary tumor (*16*).

The plasma-cell tumor in the mouse has many differences and some remarkable similarities to multiple myeloma in man. Like myeloma, the plasma-cell neoplasm occurs characteristically in old animals. Other similarities are the effect on the bone shown by one line, in which a characteristic "punched-out lesion" was produced, and on the kidney in the other line, in which "giant cells" similar to those in the "myeloma kidney" appeared. The morphology of the kidney lesion in the mouse supports the opinion that the giant cells in the "myeloma kidney" result from a fusion of tubular cells, as claimed by Allen (*17*), and are not a foreign-body cell formation. Failure to find Bence Jones protein in the mice bearing the 2 transplantable neoplasms may be due to the failure of the neoplastic cells to undergo the particular alteration necessary for the production of

this abnormal product. Bence Jones protein is not regularly present in multiple myeloma and it has not been reported in extramedullary plasmacytomas. The Bence Jones protein may never appear in the mouse because of a species difference. The similarity of the electrophoretic pattern in one of the transplant lines to the pattern in some cases of multiple myeloma is striking and indicates a chemical as well as morphologic similarity (*9*).

The site of origin of plasma-cell neoplasms in the gastrointestinal tract in the strain C3H mouse is unlike the origin of the tumor in man. Nearly all plasma-cell tumors in man originate in bone marrow. There may be a parallel, however, in the occasional plasmacytomas developing in the upper respiratory tract, which is the predominant site in extramedullary neoplasms (*11*). Human cases have not shown the predilection for infiltration of neoplastic cells in the ovaries that was shown by 70429. A Russell-body type modification has been reported in extramedullary plasmacytomas in man (*11*). Like myeloma, in which a considerable range of cytologic variation from the normal plasma cell is reported, one transplanted line showed a striking morphologic resemblance to normal plasma cells and the other was undifferentiated. Although there was variability in clinical behavior in the primary tumors once the tumor lines were stabilized, the mice bearing the transplanted tumor died at approximately the same time, indicating that the outcome depended upon the characteristics of the injected cell and was not caused by degrees of resistance in the host. Human cases of myeloma also show a remarkable range in survival time. A given patient may die a few weeks after the disease is diagnosed and another may live for several years.

Summary

A number of plasma-cell neoplasms originating at the ileocecal area in strain C3H mice have been described. These tumors have been found most often in old mice, many of which were untreated. The degree of differentiation in the neoplastic cells differed widely, and the tumors varied in extent from a small local lesion at the ileocecal junction to a widespread neoplasm involving lymph nodes and viscera.

Two lines of transplanted plasma-cell neoplasms were established. In one line the cells were morphologically undifferentiated, while in the other line the cells had characteristic features of normal plasma cells. In the undifferentiated line a Russell-body type modification in the tumor cells was observed, and a kidney lesion was rarely found. In mice bearing the more differentiated neoplasm, an osteolytic lesion and changes in plasma proteins such as occur in multiple myeloma in man were found.

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PLATE 19

FIGURE 1.—Strain C3H mouse, 14 months old. Received gastric instillation of dibenzanthracene. Small plasma-cell lesion near the ileocecal junction. Intestinal wall is thickened and infiltrated by plasma cells. Mucosa is ulcerated at one point. Hematoxylin and eosin. × 50

FIGURE 2.—High magnification from same lesion to show cell detail. Cells show usual features of plasma cells, such as eccentric nucleus, deeply stained cytoplasm, and clear central area near nucleus. Giemsa stain. × 950

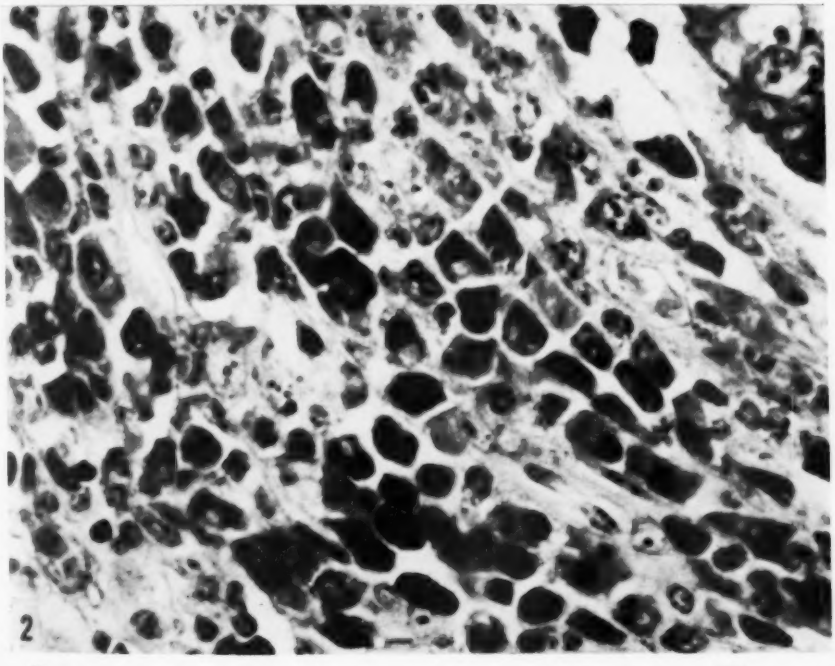

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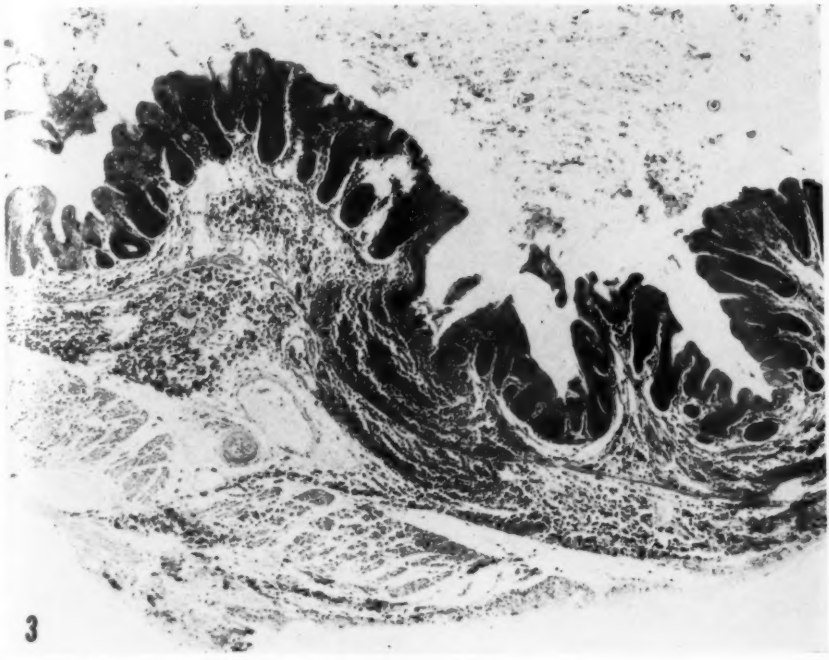
PLATE 20

FIGURE 3.—Strain C3H mouse, 26 months old, injected with methylcholanthrene when 6 weeks old. Small plasma-cell lesion located in ileocecal area. Mucosa is ulcerated at one point. Necrotic base of ulcer contains many granulocytes. Plasma cells extend through intestinal wall in direction of mesenteric node. Giemsa stain. × 65

FIGURE 4.—Strain C3H mouse, 14 months old, injected with methylcholanthrene when 15 days old. Original plasma-cell neoplasm of line 70429. Lower end of ileum was ulcerated for a distance of 0.5 cm. Neoplastic plasma cells have infiltrated mucosa and muscle wall, and masses of neoplastic cells are present in the mesentery. Hematoxylin and eosin. × 52



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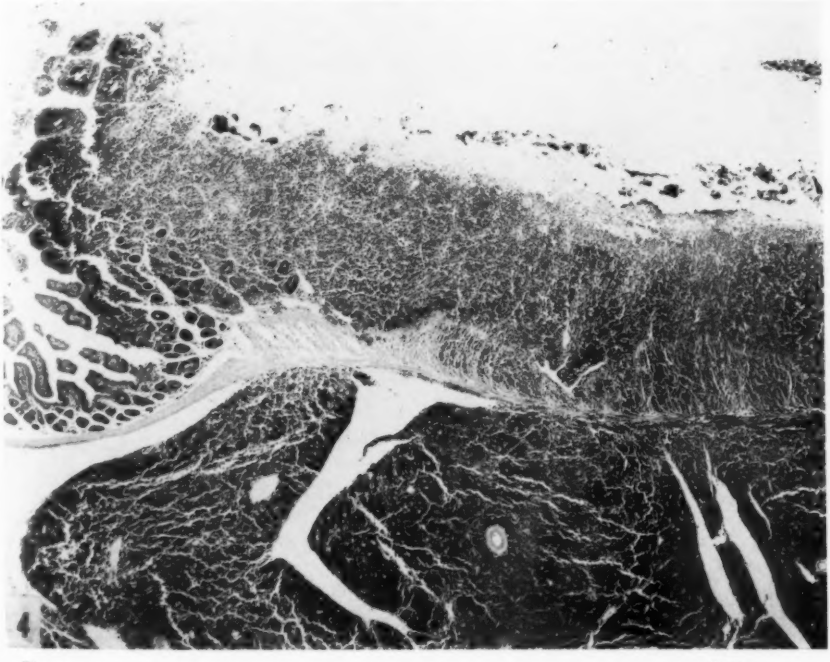

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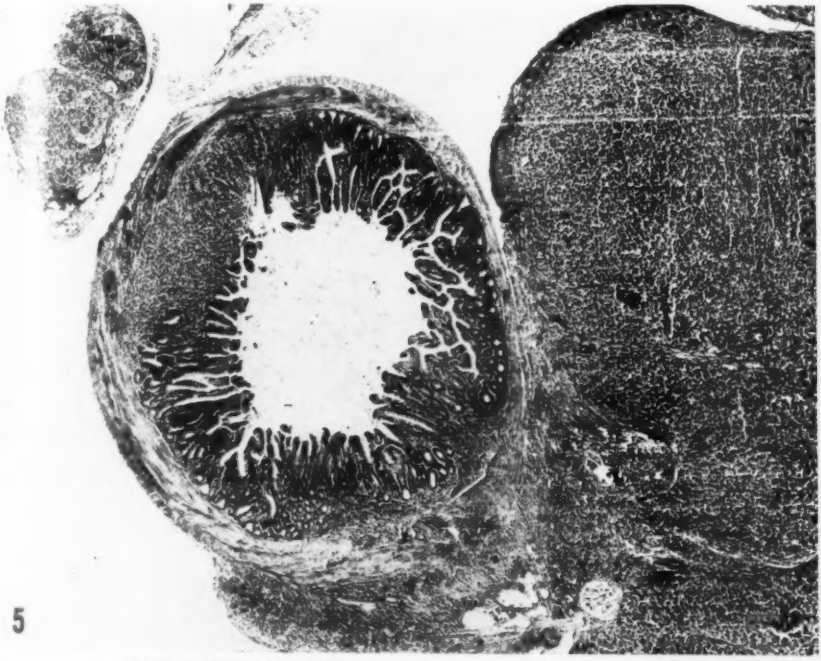
PLATE 21

FIGURE 5.—Strain C3H mouse, 24 months old, gonadectomized at 2 months; donor of plasma-cell neoplasm line X5563. Cross-section from lower end of ileum showing an area of ulceration and extension of tumor tissue into the mesentery. Hematoxylin and eosin. × 21

FIGURE 6.—Another section of the same tumor showing involvement of cecum. Plasma cells in this tumor were well differentiated. Hematoxylin and eosin. × 21



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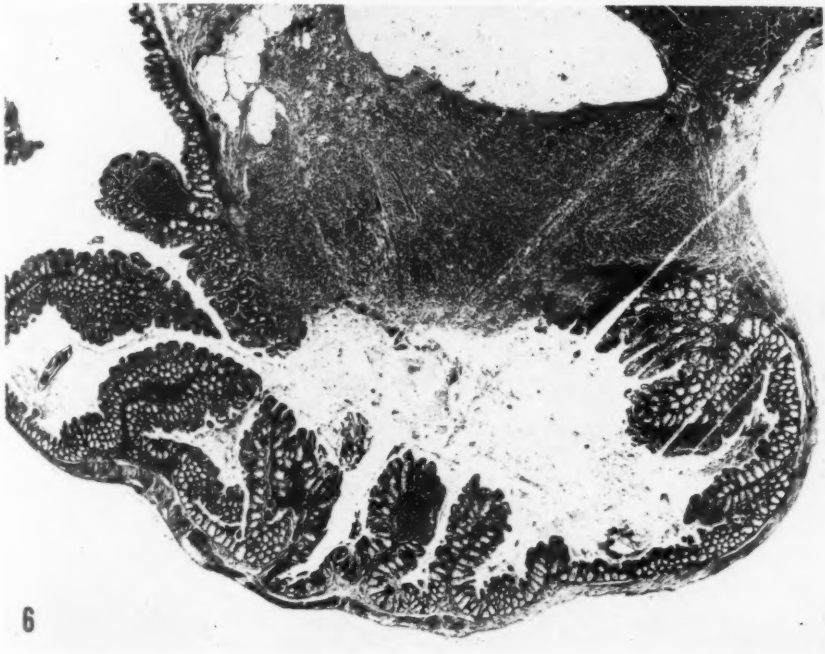

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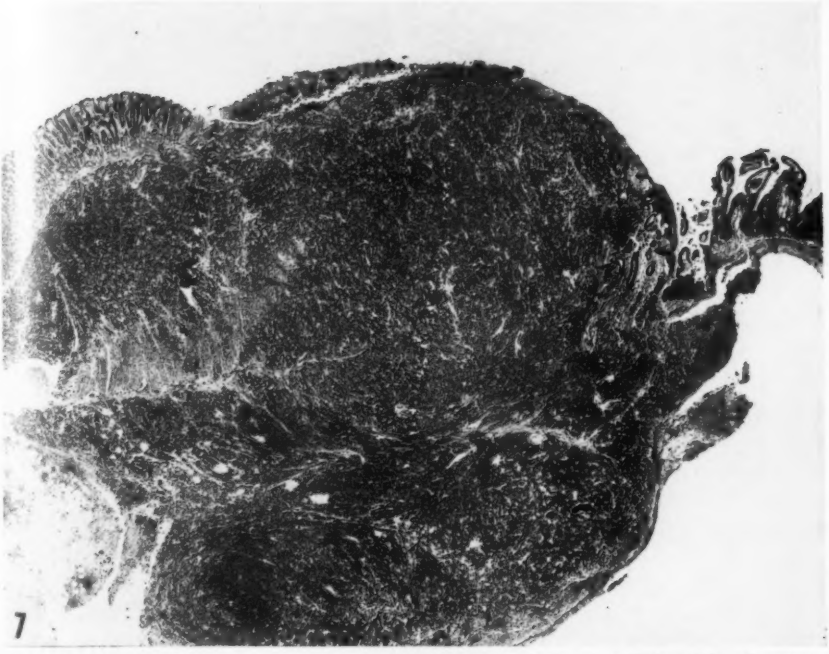
PLATE 22

FIGURE 7.—Strain C3H mouse, 1 year old, given 800 r followed by BALB/c bone marrow when 3 months old. Plasma-cell neoplasm originating at the gastroduodenal junction. Gastric mucosa is seen on *left* and mucosa of duodenum at *right*. Tumor cells have replaced normal tissue at the gastroduodenal junction, and mucosa and Brunner's glands are destroyed. Tumor cells extend into subserosal tissue. Hematoxylin and eosin. × 22

FIGURE 8.—Higher magnification of an area from the tumor shown above. On the *left* is a mass of neoplastic plasma cells. Cells in the group on the *right* are non-neoplastic. Close similarity of the large neoplastic cells to the normal cell can be seen. Hematoxylin and eosin. × 380



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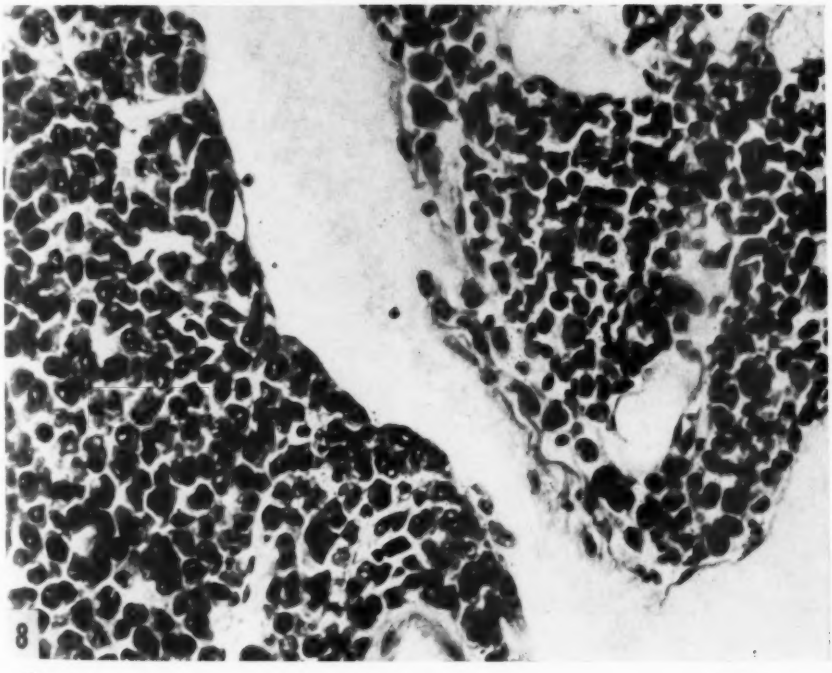

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PLATE 23

FIGURE 9.—Transplant plasma-cell tumor line 70429, generation 3. Great enlargement of the ovaries due to infiltration by plasma-cell neoplasm. × 1.1

FIGURE 10.—Generation 22. Microscopic accumulations of neoplastic cells are still common in the ovary. Normal follicles are still found at *lower right*. Hematoxylin and eosin. × 35

FIGURE 11.—**A**. Generation 27. Subcutaneous inoculation. Neoplastic cells are poorly differentiated. Pale eosinophilic cells with abundant cytoplasm and vesicular nuclei are interspersed among the plasma cells. × 420. **B**. Smear of cells from an ascites tumor showing 1 of the large pale cells and neoplastic plasma cells. Wright's stain. × 1260

FIGURE 12.—Generation 3. Modified Russell-body formation in neoplastic cells. Cells appear to be progressively enlarged by accumulation of an eosinophilic substance. Cell in *upper right* is one of the large eosinophilic cells containing basophilic particles. Hematoxylin and eosin. × 420

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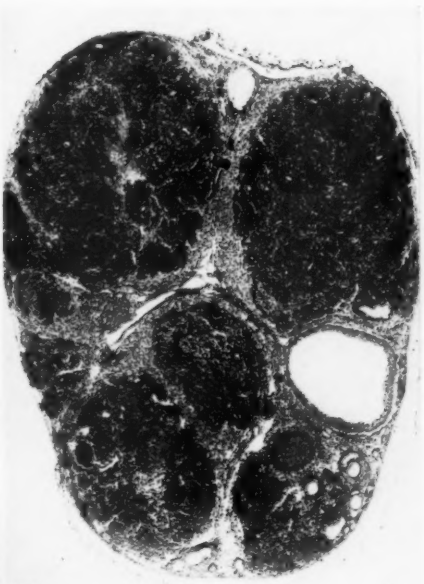
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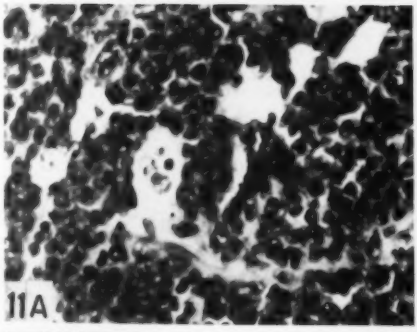
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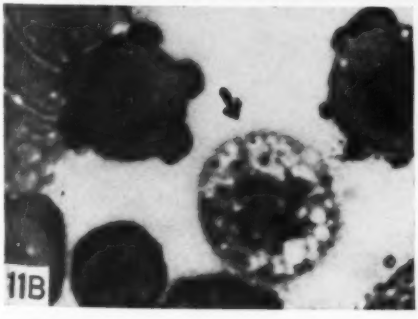
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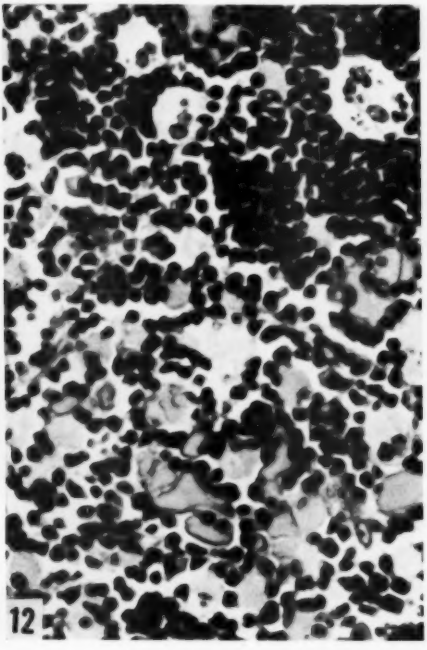
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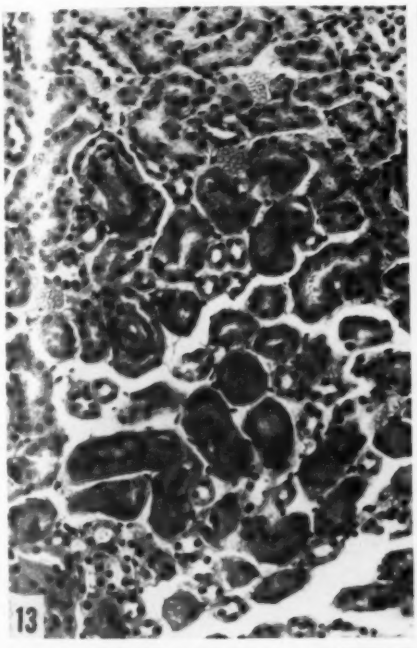
PLATE 24

FIGURE 13.—Transplant-line 70429, generation 14. Kidney lesion. Syncytial masses of cells can be found within tubules. Hematoxylin and eosin. × 200

FIGURE 14.—High magnification of a tubule shown in figure 13. Syncytial mass of cells is seen within the lumen. Hematoxylin and eosin. × 735

FIGURE 15.—Transplant-line X5563, generation 1. Detail of neoplastic plasma cells. Eccentric position of nucleus and a pale central cytoplasmic area show clearly. Occasional cells are binucleate. Hematoxylin and eosin. × 735

FIGURE 16.—Transplant-line X5563, generation 3. Destruction of bone by neoplasm. Longitudinal section of sternum. Normal marrow cells are replaced by tumor cells. Bone is interrupted at 2 points on *right*. Hematoxylin and eosin. × 70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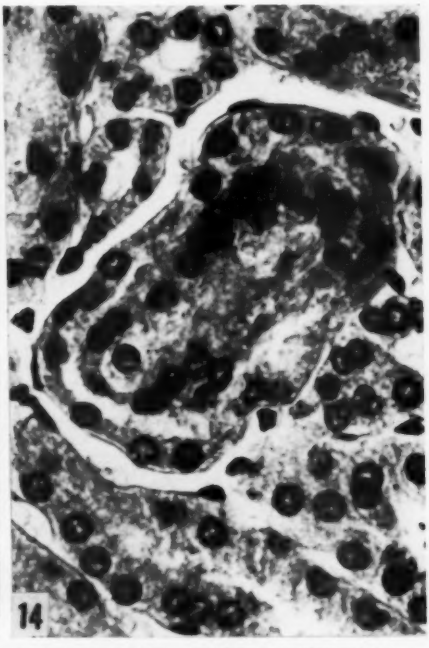
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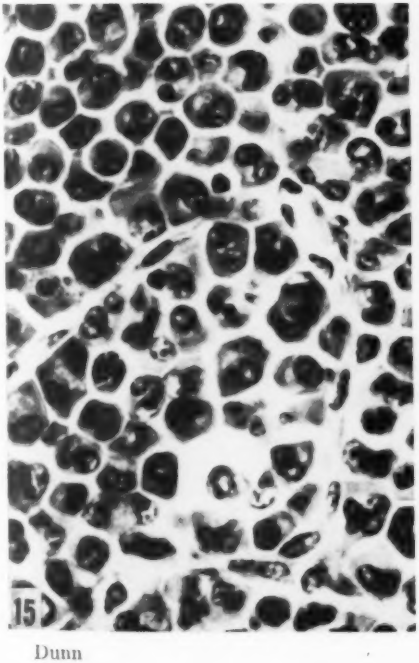
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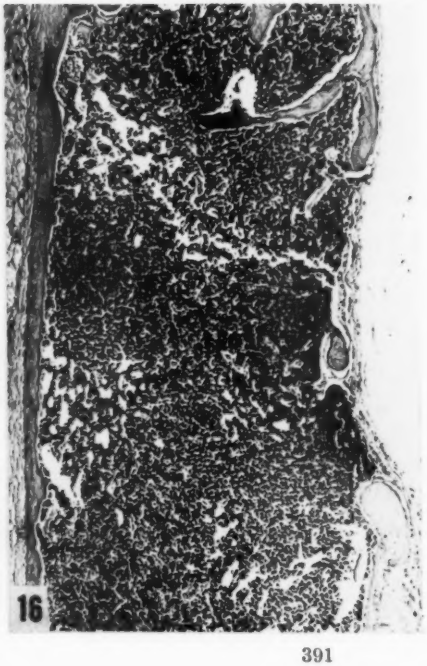
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A Study of Protein Synthesis in Tissue Culture [1]

JOSEPH F. MORGAN *and* MAUREEN A. MCCRONE,
Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada

Recent studies from this laboratory (*1*) have shown that a variety of naturally occurring substances, when incorporated in synthetic medium M 150 (*1*, *2*), markedly prolong the survival of chick embryonic-heart cultures. During these investigations, it was observed that high concentrations of certain materials, such as embryo extract and yeast extract, caused greatly increased areas of proliferation, followed by rapid death of the cultures. Since this phenomenon does not appear to have been described previously, an attempt was made to correlate the observed effects with the protein content of the tissue cultures. For these experiments, the total-protein method of Lowry and coworkers (*3*), which has been used in this laboratory for the past 3 years to standardize chick embryonic-heart cultures (*4*, *5*), was selected. The results reported in the present communication show that certain naturally occurring materials cause an immediate rise in total protein of the cultures, followed by a subsequent decline in protein. The distribution of this activity was found to be distinctly different from that of the survival-promoting activity in the same materials.

Materials and Methods

Culture Procedures and Media

All cultures were prepared from the heart muscle of 11-day-old chick embryos. The tissue was chopped to a fine pulp and moistened with a few drops of synthetic medium, and portions were transferred to 18 × 150 mm. pyrex test tubes. The tissues were cultivated directly on the glass surface, without the use of plasma clots, in completely synthetic medium M 150 (*1*, *2*) by the conventional roller-tube technique (*6*). After an initial 7-day period, the control medium (M 150) was removed, and the cultures received the experimental media, which were renewed once a week until the experiment was terminated. Each experiment employed 80 to 100 individual cultures, prepared at the same time from a large pool of chick-embryo heart tissue. Groups of 10 to 20 cultures were used

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with each concentration of natural material tested, and all cultures were examined microscopically at frequent intervals.

Synthetic medium M 150 was prepared as described previously (*1*, *2*), and to it were added graded concentrations of Bacto yeast extract, chick-embryo extract, Bacto beef extract, whole-liver extract (Sigma), liver-fraction L (Nutritional Biochemicals) or liver coenzyme concentrate (Armour). These media were prepared every 2 weeks and were sterilized by passage through UF fritted-glass filters. Initial experiments showed that the tyrosine content of medium M 150 (40.0 mg. per l.) interfered with the subsequent protein determinations, even after repeated washing of the cultures. It was found experimentally that the tyrosine level of medium M 150 was greater than the concentration necessary to maintain optimal survival of the cultures. Reduction of the tyrosine level to one tenth its normal concentration in medium M 150 (medium M 708) did not decrease significantly the survival time of the cultures and eliminated interference by this amino acid during the subsequent protein determinations. Additional experiments with nutritionally depleted cultures (*5*) have shown that maximal culture survival is supported by tyrosine concentrations between 1.0 and 10.0 mg. per l. (*7*). Based on these data, the new medium—M 708—containing 4.0 mg. per liter of L-tyrosine, was used throughout the protein experiments.

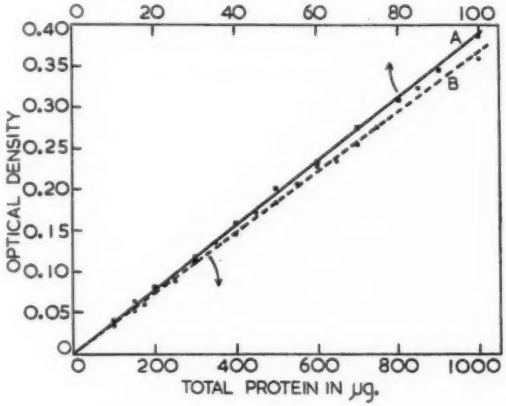
All glassware was heated to 90° C. in strong nitric acid (1 part concentrated nitric acid plus 2 parts water) and rinsed numerous times in distilled and, finally, ion-exchange water. After this washing procedure, extraction of the glassware with 0.1 *N* sodium hydroxide produced no demonstrable color reaction with the Folin reagent.

Protein Determination

Total protein was determined colorimetrically following reaction with copper and the Folin reagent (*8*), according to the procedure of Lowry and coworkers (*3*). The alkaline protein solution was mixed with alkaline copper sulfate and allowed to stand at room temperature for 15 minutes. The Folin reagent was added and the tubes were mixed immediately and allowed to stand at room temperature for 30 minutes. At the end of this time, the color intensity was measured in a spectrophotometer (Coleman Junior) at 700 mμ. The optimal reaction time with alkaline copper, optimal dilution of the concentrated Folin reagent, optimal period for maximum color development, and absorption maximum of the colored complex were all verified experimentally. Determinations were normally carried out in duplicate, except in the preparation of the standard curves, for which quadruplicate measurements were made. Aliquots of 0.1 *N* sodium hydroxide were carried through the entire procedure to provide blank values with each set of determinations.

The relationship between color intensity and protein concentration was determined, using 2 types of reference material: (*a*) Crystalline bovine serum albumin (Armour) was dissolved in 0.1 *N* sodium hydroxide, and graded levels were used for the preparation of a standard curve

(text-fig. 1, curve A). A linear relationship was found between color intensity and protein concentration over the range 10 to 100 μg. At higher concentrations, deviation from linearity was observed. (*b*) The hearts were removed from six 11-day-old chick embryos, the large blood vessels were cut away and the hearts were cut into 4 portions. The tissue was rinsed quickly in balanced salt solution, blotted dry on filter paper, weighed, and dissolved, with mechanical shaking, in 0.1 *N* sodium hydroxide. Graded aliquots of this solution were then used for the preparation of a standard curve (text-fig. 1, curve B). A linear relationship is apparent between color intensity and protein concentration over the range 100 to 1000 μg. wet tissue protein. At concentrations above 1000 μg., deviation from linearity was observed.



TEXT-FIGURE 1.—Relationship between color intensity and protein concentration by the Lowry method. Curve A—standard curve based on crystalline bovine serum albumin (Armour); curve B—standard curve based on wet weight of chick embryonic-heart tissue.

Good correlation is apparent between the standard curve based on crystalline serum albumin (curve A) and the curve based on wet weight of heart protein (curve B). For all practical purposes, the curves may be considered as identical throughout most of the concentration range. Optical-density readings may therefore be converted to either wet weight of heart protein or dry weight of crystalline albumin by use of the factor 10. From these results, it was concluded that the method was suitable for measuring total protein in tissue cultures and that the values obtained could be expressed as either wet weight of heart tissue or dry weight of protein.

For measurement of total protein in the experimental tissue cultures, groups of tubes were selected at appropriate time intervals, the synthetic medium was removed, and an equal volume (1.5 ml. per tube) of Hanks' balanced salt solution (*9*) was added. The cultures were replaced in the slowly rotating drums for a period of 15 minutes; the Hanks' solution

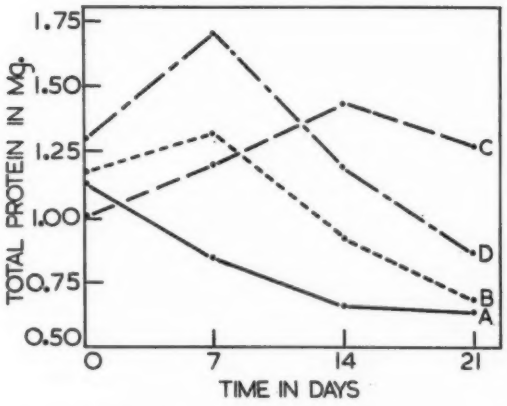
was then removed by aspiration and discarded. This washing procedure was carried out 3 times to remove adsorbed material. To each tube was added 3.0 ml. of 0.1 *N* sodium hydroxide, previously warmed to 50° C.; the tubes were shaken vigorously by hand for 2 to 3 minutes, restoppered, and placed in a rapidly rotating drum (2000 r.p.h.) for 3 days. At the end of this time, the tubes were agitated vigorously in a reciprocating shaker for 30 minutes to dissolve the last traces of tissue protein. Aliquots of the resulting solution (diluted to contain approximately 50 μg. per ml.) were used for the protein determinations. It was found that this procedure, although rigorous, was necessary to dissolve the tissue protein completely. The use of sodium hydroxide solutions more concentrated than 0.1 *N* or temperatures above 50° C. resulted in loss of protein through hydrolysis.

Extensive tests were made to determine whether major loss of protein from the cultures occurred during the repeated washing with Hanks' solution. Microscopic examination of marked areas in the culture tubes before and after the washing procedure failed to reveal gross loss of tissue particles. Paper chromatography of the wash solutions (*5*) showed the presence of amino acids, but no protein spot was detected. From these experiments, it was concluded that loss of protein from the cultures during the washing procedure was negligible.

The variability of the method was tested by measuring the total protein content of 44 individual cultures selected at random from a set of 100 cultures prepared from a large tissue pool. A mean protein value of 1837 μg. was obtained, with a standard deviation of ±302 μg. While the mean protein value was slightly different for each set of 100 cultures, the inclusion of unsupplemented M 150 in each experiment served as an internal control. Thus, minor variations in initial protein content did not significantly affect the experimental results. From these observations, it was concluded that the protein level of 1 to 2 mg. wet weight per culture, normally used in cell-nutrition studies (*1*, *4*, *5*), was adequate for the protein investigations.

Results

Effect of Bacto yeast extract on protein content of chick embryonic-heart cultures.—Since yeast extract had been observed previously (*1*) to have marked effects on culture survival, it was selected as the first material to be tested for activity on the protein content of the cultures. Sets containing 100 cultures were prepared in the usual manner and supplied with 0.75 ml. of medium M 150 for the initial 7-day period. At the end of this time, the cultures were examined microscopically and any unsuitable ones discarded (*1*, *2*, *4*); all remaining cultures received 1.5 ml. of M 150 containing the test level of yeast extract. Immediately, and at 7, 14, and 21 days thereafter, 20 cultures were selected at random and the total protein content was determined. The effect of graded yeast-extract concentrations on the protein content of the cultures during this 21-day period is shown in text-figure 2.



TEXT-FIGURE 2.—Effect of Bacto yeast extract on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.01 percent yeast extract; curve C—0.1 percent; curve D—1.0 percent. All concentrations of yeast extract were incorporated in medium M 150. Each point represents the mean value based on 20 individual cultures.

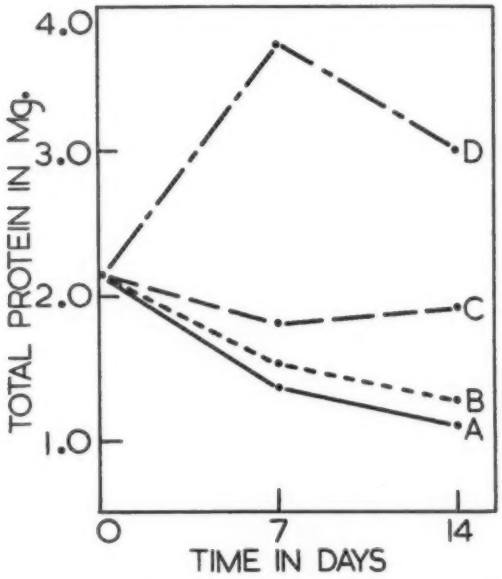
Cultures in the control medium, M 150 (curve A), show a progressive drop in protein content. This decrease is greatest during the first 14 days and then begins to level off. Between 14 and 21 days little, if any, decline in protein content may be seen. Incorporation of 1.0 percent yeast extract in the medium (curve D), results in a marked increase in total protein during the first 7 days of cultivation, followed by a rapid decline to nearly the control level by 21 days. When 0.1 percent yeast extract is incorporated (curve C), a steady increase in protein content during the first 2 weeks is observed, followed by a decline during the 3rd week of the experiment. This is the only concentration of yeast extract at which the protein content of the cultures remained higher than the initial value for a prolonged period. At 0.01 percent yeast extract (curve B), a slight increase in protein during the first 7 days may be seen and then a decrease to approximately the control level by 21 days. Concentrations of yeast extract below 0.01 percent failed to increase the protein content of the cultures and yielded response curves indistinguishable from the curve for the control medium.

From these results, it was apparent that yeast extract exerted a major effect on the protein content of the tissue cultures and that the magnitude of the effect could be related directly to the yeast-extract concentration. Since the majority of the effects were observed during the first 14 days of cultivation, it was concluded that subsequent experiments could be limited to a 2-week period. Statistical analysis of the data showed that 10 cultures were sufficient to provide valid information at each time interval tested. Consequently, all subsequent experiments were based on groups of 10 cultures over a 2-week period. This arrangement made it possible to test 3 levels of a biologically active material, plus a control

series, within a single set of 100 cultures. In this way, variation in the amount of tissue protein initially present and, consequently, variations in the starting point of curves A, B, C, and D (text-fig. 2) were eliminated.

Effect of graded levels of chick-embryo extract.—Chick-embryo extract was prepared by the procedure normally used in this laboratory (*10*), and graded concentrations were incorporated in medium M 150. The effect of these media on the protein content of chick embryonic-heart cultures is shown in text-figure 3. It is evident that, as in the case of the previous experiments with yeast extract, three has been a progressive decrease in the protein content of the cultures maintained in the control medium M 150 (curve A). The addition to the medium of 10.0 percent chick-embryo extract has caused the protein content of the cultures to nearly double in 7 days (curve D). After this period, a sharp decline in protein content is apparent. The presence of 1.0 percent embryo extract (curve C) has resulted in a slight drop in protein during the first 7 days, followed by a gradual rise to nearly the initial level. At 0.1 percent (curve B) and concentrations below this level, the responses obtained could not be distinguished from the response with the control medium (curve A).

A statistical evaluation of the effect of graded levels of chick-embryo extract on the protein content of the tissue cultures is presented in table 1. During cultivation of the tissues in unsupplemented medium M 150,



TEXT-FIGURE 3.—Effect of chick-embryo extract on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.1 percent embryo extract in M 150; curve C—1.0 percent; curve D—10.0 percent. Each point represents the mean value based on 10 individual cultures.

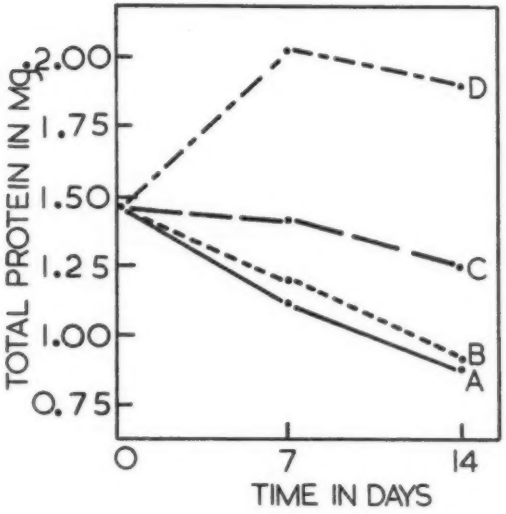
total protein declines progressively and, by 14 days, only 50 percent of the initial protein level remains (columns 4 and 5). The rate of protein loss decreases progressively with the addition of increasing levels of chick-embryo extract. When 10 percent chick-embryo extract is incorporated in M 150, loss of protein is prevented completely and a protein increase of approximately 75 percent is obtained within the first 7 days of cultivation. This protein increase subsequently declines to approximately 40 percent by the 14th day of cultivation. This level of chick-embryo extract was the only concentration found capable of stimulating net protein synthesis under the present conditions. Calculation of the percentage increase in total protein at 7 and 14 days, in terms of the protein content of the cultures maintained in unsupplemented M 150 for the same length of time (columns 6 and 7), emphasizes the magnitude of the effect exerted by embryo extract. The ability of 10 percent embryo extract to stimulate over-all protein synthesis is clearly demonstrated again, and it is of interest to note that this relative increase in total protein remains essentially constant at approximately 175 percent throughout the entire 14-day test period. Although the large standard deviations (columns 2 and 3) indicate considerable variation in the protein content of individual cultures, it is apparent that this method provides a suitable analytical tool for detecting major effects on the protein content of chick embryonic-heart cultures. A statistical evaluation of other natural materials tested produced similar results.

TABLE 1.—*Statistical evaluation of effect of chick embryonic extract on protein content of chick embryonic-heart tissues cultivated in synthetic medium M 150**

Embryo-extract concentration (*percent*)	Total protein in μg.†		Difference from zero time (*percent*)		Increase over M 150 control (*percent*)	
	7 days	14 days	7 days	14 days	7 days	14 days
0.0(M 150)	1360 ± 412	1094 ± 247	−36. 8	−49. 1	—	—
0.1	1529 ± 599	1312 ± 347	−28. 9	−39. 0	12. 4	19. 9
1.0	1838 ± 637	1955 ± 461	−14. 5	−9. 1	35. 1	78. 7
10.0	3752 ± 548	3039 ± 821	+74. 4	+41. 3	175. 9	177. 7

* Control cultures selected at random at zero time contained 2151 ± 604 μg. protein per tube.
† Mean value and standard deviation have been calculated in each case from groups of 10 cultures.

Effect of graded levels of Bacto beef extract.—Graded levels of beef extract, over the concentration range previously shown to be effective in prolonging culture survival (*1*), were incorporated in medium M 150 and the effect on total protein of the cultures was determined. The results of these experiments are shown in text-figure 4. The highest concentration tested, 0.64 percent (curve D), has caused a considerable increase in total protein during the first 7 days of cultivation, followed by a gradual decrease after this period. The next lowest concentration, 0.16 percent (curve C), maintained the protein level without change for 7 days, but after this time a gradual decline was evident. Lower concentrations (curve B) showed the same general pattern of protein decrease exhibited



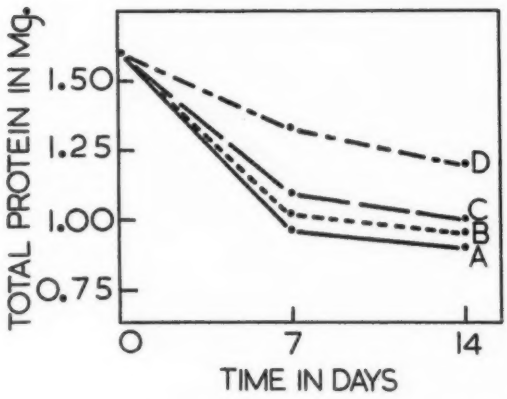
TEXT-FIGURE 4.—Effect of Bacto beef extract on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.04 percent beef extract in M 150; curve C—0.16 percent; curve D—0.64 percent. Each point represents the mean value based on 10 individual cultures.

by the cultures in the control medium (curve A). Concentrations of beef extract higher than 0.64 percent could not be tested because of the limited solubility of the material in M 150 and the difficulty of passing the gummy materials through fritted-glass filters.

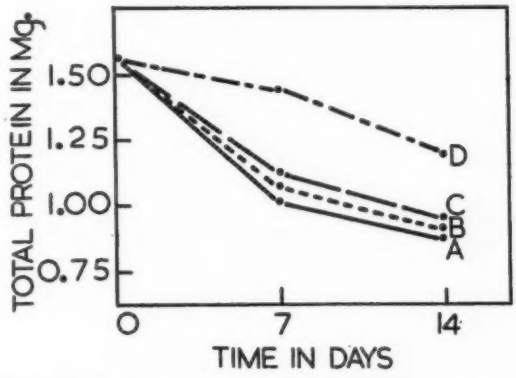
Effect of various liver fractions.—The effect of graded concentrations of whole-liver extract (Sigma) on protein content of the cultures is illustrated in text-figure 5. It is evident that only the highest level tested, 0.1 percent (curve D), showed any appreciable ability to arrest the decrease in total protein resulting from cultivation in the control medium (curve A). Concentrations of whole-liver extract of 0.001 percent (curve B) and 0.01 percent (curve C) were completely ineffective. Concentrations higher than 0.1 percent were not tested, since this level had been shown previously to represent the upper limit of activity for the survival of chick embryonic-heart cultures (*1*).

Concentrations of liver-fraction L (Nutritional Biochemicals) similar to those of whole-liver extract were tested for their effect on the protein content of the cultures, with the results shown in text-figure 6. As in the case of the whole-liver extract, only the highest level of liver-fraction L tested (0.1%, curve D) had any significant effect on the protein content of the cultures. At this concentration, the rate of decrease of protein was appreciably less rapid than with the unsupplemented control medium (curve A).

The effect of graded concentrations of liver coenzyme concentrate (Armour), at the same levels as in the case of the 2 other liver preparations,

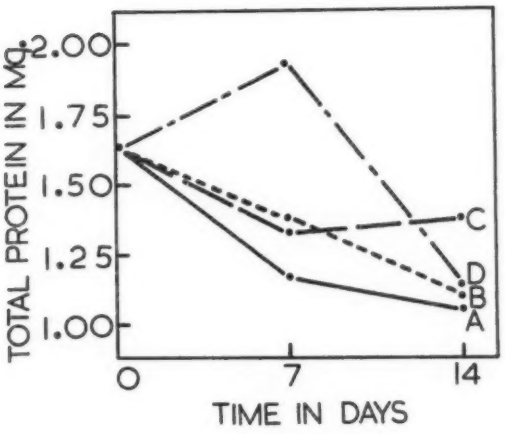


TEXT-FIGURE 5.—Effect of whole-liver extract on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.001 percent liver extract in M 150; curve C—0.01 percent; curve D—0.1 percent. Each point represents the mean value based on 10 individual cultures.



TEXT-FIGURE 6.—Effect of liver-fraction L on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.001 percent fraction L in M 150; curve C—0.01 percent; curve D—0.1 percent. Each point represents the mean value based on 10 individual cultures.

is shown in text-figure 7. The highest level tested (0.1%, curve D) caused a marked increase in protein content during the first week of cultivation. This was followed, in turn, by a rapid decrease in total protein, so that by the end of the second week the protein value coincided with that of cultures in the unsupplemented medium (curve A). At a lower concentration of coenzyme concentrate (0.01 percent, curve C), the protein content of the cultures decreased considerably during the first week and then increased slightly during the second week. At the lowest level of coenzyme concentrate tested (0.001%, curve B), the decrease in total protein during the first week was somewhat less than that occurring in the



TEXT-FIGURE 7.—Effect of liver coenzyme concentrate (Armour) on protein content of chick embryonic-heart cultures. Curve A—medium M 150 control; curve B—0.001 percent coenzyme concentrate in M 150; curve C—0.01 percent; curve D—0.1 percent. Each point represents the mean value based on 10 individual cultures.

control medium (curve A), but by the end of the second week the protein content of both groups of cultures was almost identical.

Relative activity of natural materials tested.—The relative ability of natural materials to stimulate protein formation in chick embryonic-heart cultures is summarized in table 2. The greatest percentage increase in protein content was effected by chick-embryo extract, followed, in order of decreasing activity, by beef extract, yeast extract, and liver coenzyme concentrate. When the order of relative activity was based on the concentration necessary for maximal stimulation, an entirely different sequence was obtained. The most active substance then appeared to be liver coenzyme concentrate, followed, in order of decreasing activity, by beef extract, yeast extract, and chick-embryo extract. When the lowest concentrations able to cause protein increase at either 7 or 14 days were compared, liver coenzyme concentrate and yeast extract appeared to

TABLE 2.—*Relative ability of natural materials incorporated in synthetic medium M 150 to stimulate protein formation in chick embryonic-heart cultures*

Substance tested	Increase in total protein at 7 days (*percent*)	Concentration for maximal stimulation (*percent*)	Lowest active concentration* (*percent*)
Chick-embryo extract	74. 4	10. 0	1. 0
Beef extract	38. 0	0. 64	0. 16
Yeast extract	30. 3	1. 0	0. 01
Liver coenzyme concentrate	18. 1	0. 1	0. 01
Whole-liver extract	0	0	0
Liver-fraction L	0	0	0

*This figure represents the lowest concentration able to cause protein increase at either 7 or 14 days.

have the same relative activity, followed by beef extract and chick-embryo extract.

Discussion

The present studies were undertaken to determine the effect of various naturally occurring materials on the protein content of chick embryonic-heart cultures and to correlate these findings with previous observations on tissue-culture nutrition and metabolism. The total-protein method of Lowry and coworkers (*3*) was selected for these studies because of its simplicity and high degree of sensitivity. Detailed investigation of this method has shown that color intensity is linear between protein concentrations of 10 and 100 μg. with crystalline serum albumin and between 100 and 1000 μg. with wet weight of heart tissue. A possible disadvantage of the Lowry method is the danger of protein hydrolysis under the conditions required to dissolve the tissue cultures. Alkaline copper tartrate has been used to dissolve cell-strain cultures (*11*) and 1.0 *N* sodium hydroxide has been employed for various chick embryonic tissues (*12*). In the present work, comparative studies with crystalline serum albumin dissolved by warm 0.1 *N* sodium hydroxide and by gentle agitation in this solution without warming showed only negligible differences in protein content. It was concluded, therefore, that the procedure found necessary in the present studies could be applied to tissue cultures without serious loss of protein by hydrolysis.

Although extensive studies have been made recently on tissue-cell nutrition and metabolism, relatively little attention has been given the problem of protein synthesis in tissue cultures. Gerarde, Jones, and Winnick (*13*) found no net increase of protein in large-scale cultures of chick-embryo heart, lung, or intestine maintained in synthetic media containing various levels of total amino acids. This observation is confirmed by the results of the present experiments since chick-embryo heart tissue cultivated in synthetic medium M 150 showed a progressive decline in protein content. The decrease in protein was most extensive during the first 7 days of cultivation and represented a decline to approximately 65 percent of the original level within that period. During subsequent cultivation, the decrease in total protein continued at a slower rate.

An apparent decrease in total protein might conceivably be attributed to the loss of discrete fragments from the freshly explanted tissues during the cultivation period. To avoid this possibility in the present experiments, the fragments were made as small as possible by protracted chopping of the heart tissue before explantation. Previous experiments on culture survival (*1*, *4*, *5*) had shown that the greatest loss of tissue fragments occurred during the initial 7 days of cultivation. Accordingly, all cultures for the protein studies were kept in medium M 150 for a pre-experimental period of 7 days, and any cultures with loose tissue fragments were discarded before the experimental media were added. Protein measurements were then made over the following 2- to 3-week period, when culture uniformity was greatest, as shown by microscopic observations. The extent of protein loss through secretion of soluble protein into the

culture medium remains an unknown factor, although the addition of protein-precipitating agents, such as sulfosalicylic acid, to the used culture medium failed to produce demonstrable turbidity. From these considerations, it was concluded that the decrease in total protein observed under the present conditions resulted from a rate of protein synthesis insufficient to balance protein degradation. This conclusion is supported by the observations of Gerarde, Jones, and Winnick (*13*), who found no net increase of protein in cultures maintained in synthetic media, even though loss of tissue fragments was prevented by embedding the cultures in a plasma clot.

The same authors (*13*) have shown that 25 percent embryo extract caused a 100 percent increase in protein in chick-embryo heart cultures, but they did not continue their observations over a prolonged time period. In the present work, a similar initial result has been shown (table 1) but this was followed by a rapid decrease in protein during subsequent cultivation. These results suggest that protein formation has been stimulated initially by some unidentified component or components of the embryo extract. In the presence of a medium inadequate to support continued rapid protein synthesis, overstimulation of the cultures appears to have created a general nutritional deficiency, resulting in rapid degeneration and concomitant loss of total protein. Previous studies from this laboratory (*14*) have shown that maximal survival of chick embryonic-heart cultures is obtained when the synthetic medium is renewed once a week. Renewal of the nutrient medium 2 or 3 times each week brought about a considerable reduction in survival time. For this reason, and in order to make the present study comparable with the previous survey of natural materials (*1*), a schedule was followed in which the medium was changed once each week. The results obtained in the present investigation suggest that creation of a nutritional deficiency through overstimulation of the cultures may provide a sensitive system for detecting nutritional requirements that are not revealed by other methods. Further experiments of this nature are now being planned.

The ability to stimulate protein formation in tissue cultures was not restricted to embryo extract but was also exhibited by yeast extract, beef extract, and liver coenzyme concentrate, although not by whole-liver extract or liver-fraction L (table 2). The greatest percentage increase in protein formation was observed in the presence of chick-embryo extract, a result that is in agreement with the well-known ability of this material to stimulate proliferation in tissue cultures (*15*). It should be noted, however, that a relatively high concentration of chick-embryo extract was required to exert this effect. On the basis of effective concentration, liver coenzyme concentrate, beef extract, and yeast extract were considerably more active than chick-embryo extract. The inactivity of whole-liver extract and liver-fraction L was somewhat surprising in view of the marked stimulation observed with low concentrations of liver coenzyme concentrate and the ability of whole-liver extract and fraction L to prolong the survival of tissue cultures (*1*). It appears that crude liver extracts contain

both stimulatory and inhibitory substances and that the inhibitory substances are removed during preparation of the coenzyme fraction. It was observed with each active material that stimulation of protein synthesis was followed by rapid protein loss, an effect that to our knowledge has not been reported previously with tissue cultures.

A major criticism of the use of fresh tissue explants for cell-nutrition studies has been the lack of suitable criteria for short-term proliferation. Cell-survival studies, based on medium M 150 or modifications of this medium, involve experiments of 40 to 60 days' duration, and this protracted time period has handicapped attempts to isolate the active ingredients in natural materials. As a consequence, short-term assays have been sought by many workers. Enumeration of cell nuclei (*16*) provides a precise and quantitative method of evaluating cell multiplication, and measuring packed-cell volume (*17*) has been advocated as a rapid method of detecting major population changes. Unfortunately, these methods, while suitable for use with cell-strain cultures, are difficult to apply to the multilayer system existing in fresh tissue explants. Oyama and Eagle (*11*) have stated that total-protein determinations can be correlated with cell counts in cell strain cultures. However, it should be emphasized that such a relationship is based on the assumption that the total-protein content per cell remains constant throughout the growth cycle, and this assumption has not been verified experimentally.

The present work indicates that protein formation and culture survival are not always related. Although many substances, such as chick-embryo extract, yeast extract, and beef extract, stimulated both protein formation and culture survival, the results obtained with liver fractions were completely contradictory. Thus, whole-liver extract and liver-fraction L, but not liver coenzyme concentrate, prolonged culture survival (*1*), while the reverse was true with stimulation of protein formation. Even when the initial results were similar, the rapid decrease in total protein, after an immediate increase, implies an overstimulation effect, possibly due to a lack of basic nutrients. These observations suggest strongly that the results of short-term protein assays must be interpreted with caution. The relationship between cell population, protein formation, and survival and the response of the cells to varying nutritional conditions are obviously very complex. The present work has shown that total-protein studies yield valuable information on the factors required for protein synthesis in tissue cultures and provide a technique suitable for fractionation studies with natural materials. However, in order to study the over-all nutritional and metabolic state of cells *in vitro*, it would seem advisable to employ protein determinations in conjunction with other measurements, such as survival.

Summary

1) Measurement of total protein by reaction with alkaline copper and the Folin phenol reagent has been shown to be a suitable method for determining protein synthesis in tissue cultures.

2) Freshly explanted chick embryonic-heart tissues cultivated in modified synthetic medium M 150 exhibit a progressive drop in total protein throughout the cultivation period. This decrease is greatest during the first week of cultivation.

3) With the use of the modified M 150 as a control medium, a survey has been made of the ability of various natural-material supplements to stimulate protein formation in chick embryonic-heart cultures.

4) The greatest percentage increase in total protein was observed after incorporation of chick-embryo extract, followed, in order of decreasing activity, by beef extract, yeast extract, and liver coenzyme concentrate.

5) When the concentrations required for maximum stimulation were the criteria, greatest activity was found in liver coenzyme concentrate, followed, in order of decreasing activity, by beef extract, yeast extract, and chick-embryo extract.

6) No ability to stimulate protein formation was found with whole-liver extract or liver-fraction L.

7) Initial stimulation of protein formation by yeast extract, chick-embryo extract, beef extract, or liver coenzyme concentrate was consistently followed by a rapid decrease of protein in the cultures.

8) The relationship of these findings to tissue-culture nutrition is discussed.

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Effects of Tumor Growth on Serum Glycoprotein Concentrations in the Rat [1, 2]

HENRY E. WEIMER, FRANCES A. QUINN, JEAN REDLICH-MOSHIN, *and* HISAKO NISHIHARA, *Department of Infectious Diseases, School of Medicine, University of California Medical Center, Los Angeles, California*

Increased concentrations of the carbohydrate-containing proteins of serums have been reported by a large number of investigators in clinical cancer. The significant data have been summarized in recent reviews (*1–3*). In contrast, few studies have been concerned with the serum glycoproteins in animals bearing transplantable tumors (*4, 5*). It seemed probable that the use of an experimental host-tumor system might provide more precise information with regard to the stage in tumor growth at which alterations in serum glycoprotein levels occurred and the relationship of tumor weight to the observed changes.

The present investigation was undertaken to study the effects of the growth of Walker carcinosarcoma 256 on the serum concentrations of total glycoprotein, the seromucoid fraction, and total protein and on the hemoglobin and hematocrit values of blood in Sprague-Dawley rats. In an attempt to determine whether the tumor might be a source of serum glycoproteins, similar estimations were made on saline extracts of lyophilized tumor tissue. For comparison with the results obtained with the lyophilized tumor tissue, serums from normal and tumor-bearing animals were dried in the frozen state and subjected to the same procedures. The influence of the site of tumor growth on the rate and extent of the alterations in serum levels of protein-bound carbohydrates was also studied.

Materials and Methods

Serial serum studies.—Adult, male Sprague-Dawley [3] rats were housed 2 per cage and maintained on a diet of Purina laboratory chow and tap water *ad libitum*. The Walker carcinosarcoma 256 was implanted subcutaneously at the midline of the back or into the thigh muscle by the methods of Talalay *et al.* (*6*).

A plot of the weight of the tumor *versus* time, in days, following implantation demonstrated a 2-phase growth: a period of slow growth of approxi-

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mately 10 days' duration—the induction period in which the tumor became established, followed by a period of very rapid linear growth. No implantation failures occurred. Animals were sacrificed at intervals following implantation by exsanguination from the heart under anesthesia. The tumor was excised and weighed. Blood was obtained from normal rats by cardiac puncture under ether anesthesia. Total serum glycoprotein, the seromucoid fraction, total serum protein, hemoglobin, and hematocrit values were determined by methods previously reported (*7*, *8*).

Tissue studies.—Following excision of the tumor, the tissue was divided into viable and necrotic sections on the basis of gross visual inspection. The tissues were washed in cold 0.14 *M* sodium chloride and minced. Tissues from 2 animals were pooled and lyophilized. The lyophilized tissues were ground to a powder in a mortar and pestle. One percent suspensions of the dried tissues were prepared in 0.14 *M* sodium chloride, *p*H 6.6. The suspensions were shaken on a Kahn shaker for 30 minutes and centrifuged at 3000 r.p.m. for 30 minutes, and the supernatant was filtered through Whatman #1 filter paper. Tissue glycoprotein, the histomucoid fraction,[4] and tissue protein were determined in the filtrates by the same methods employed for serum.

One group of animals was perfused with Tyrode's solution while under sodium pentobarbital (Nembutal®) anesthesia. The perfusing solution was introduced into the vena cava. Jugular and femoral veins were opened for drainage. Following perfusion, the tumor tissue was treated as described above.

Lyophilized-serum studies.—Serums from normal and tumor-bearing rats were lyophilized and subjected to the same procedures as the tumor tissue. The lyophilized serums were completely reconstituted in 0.14 *M* sodium chloride.

The mean, standard error of the mean, *t*, and probability values were determined by standard statistical procedures (*9*).

Results

Serial serum studies.—A summary of the results obtained with the animals that received subcutaneous implants of the tumor is presented in table 1. The first significant changes following implantation of the tumor were decreases in total serum protein and hematocrit values and an increase in the serum glycoprotein : serum protein ratio. At the end of the experimental period, at which time the weight of the tumor represented almost 20 percent of the residual carcass weight, highly significant decreases had occurred in most of the blood and serum values. Notable exceptions were the marked increases in the seromucoid fraction and in the ratios involving seromucoid, total glycoprotein : total protein and seromucoid : total glycoprotein.

Effect of site of implantation.—Data for the animals receiving intramuscular implants of the tumor are summaried in table 2. A comparison

[4] By analogy with the seromucoid fraction, the term "histomucoid fraction" is proposed for the perchloric acid-soluble, phosphotungstic acid-insoluble proteins present in tissues.

TABLE 1.—*Effects of neoplastic growth on serum glycoprotein concentrations in rats with subcutaneous tumors*

Group	A Normal	B Tumor-bearing	C Tumor-bearing	D Tumor-bearing	E Tumor-bearing
Number of animals	49	16	16	20	20
Age of tumor (*days*)	0	8	11	14	21
Initial weight (*gm.*)*	—	338 ± 7.5	313 ± 6.0	304 ± 8.7	358 ± 9.5
Final residual carcass weight (*gm.*)*	350 ± 6.9	359 ± 7.8	332 ± 6.9	345 ± 7.6	372 ± 11.7
Weight of tumor (*gm.*)*	—	0.2 ± 0.03	2.5 ± 0.53	23 ± 2.7	68 ± 9.5
Tumor weight ÷ final residual carcass weight × 100 (%)*	—	0.06 ± 0.002	0.75 ± 0.160	6.7 ± 0.69	18.3 ± 3.0
Total serum glycoprotein (*mg.%*)*	151 ± 2.1	155 ± 4.9	139 ± 5.5†	131 ± 4.4‡	138 ± 4.2‡
Seromucoid fraction (*mg.%*)*	22 ± 0.9	21 ± 0.7	23 ± 2.9	40 ± 3.0‡	54 ± 1.5‡
Total serum protein (*gm.%*)*	6.2 ± 0.06	5.9 ± 0.12†	5.5 ± 0.19‡	4.7 ± 0.12‡	4.5 ± 0.14‡
Total serum glycoprotein ÷ total serum protein × 100 (%)*	2.4 ± 0.03	2.6 ± 0.07‡	2.5 ± 0.06	2.8 ± 0.06‡	3.1 ± 0.06‡
(Total serum glycoprotein − seromucoid) ÷ total serum protein × 100 (%)*	2.1 ± 0.02	2.3 ± 0.06‡	2.1 ± 0.02	1.9 ± 0.03‡	1.9 ± 0.03‡
Seromucoid ÷ total serum glycoprotein × 100 (%)*	14.3 ± 0.45	13.5 ± 0.46	16.4 ± 1.38	30.5 ± 0.83‡	39.1 ± 0.84‡
Hemoglobin (*gm.%*)*	15.7 ± 0.25	14.8 ± 0.37	14.9 ± 0.24	12.2 ± 0.54‡	11.0 ± 0.43‡
Hematocrit (%)*	47 ± 0.4	45 ± 1.1†	45 ± 1.0†	40 ± 1.4‡	38 ± 1.4‡

*Including the standard error of the mean.
†$P = <0.05>0.01$.
‡$P = <0.01$.

TABLE 2.—*Effects of neoplastic growth on serum glycoprotein concentrations in rats with intramuscular tumors**

Group	A Normal	B′ Tumor-bearing	D′ Tumor-bearing
Number of animals	49	10	19
Age of tumor (*days*)	0	8	14
Initial weight (*gm.*)†	—	340 ± 5.0	348 ± 7.6
Final residual carcass weight (*gm.*)†	350 ± 6.9	360 ± 5.9	375 ± 8.4
Weight of tumor (*gm.*)†	—	2.5 ± 0.22	21.0 ± 0.6
Tumor weight ÷ final residual carcass weight × 100 (%)†	—	0.70 ± 0.07	5.6 ± 0.20
Total serum glycoprotein (*mg.%*)†	151 ± 2.1	(‡)139 ± 2.7§	(§)163 ± 2.6§
Seromucoid fraction (*mg.%*)†	22 ± 0.9	23 ± 1.3	(§)55 ± 1.8§
Total serum protein (*gm.%*)†	6.2 ± 0.06	(‡)5.5 ± 0.12§	(§)5.5 ± 0.07§
Total serum glycoprotein ÷ total serum protein × 100 (%)†	2.4 ± 0.03	2.5 ± 0.04	2.9 ± 0.03§
(Total serum glycoprotein − seromucoid) ÷ total serum protein × 100 (%)†.	2.1 ± 0.02	(‡)2.1 ± 0.03	1.9 ± 0.03§
Seromucoid ÷ total serum glycoprotein × 100 (%)†	14.3 ± 0.45	(§)16.2 ± 0.83	(‡)33.7 ± 0.89§
Hemoglobin (*gm.%*)†	15.7 ± 0.25	14.5 ± 0.27‡	(‡)13.4 ± 0.19§
Hematocrit (%)†	47 ± 0.4	46 ± 0.6	(§)45 ± 0.5§

*Symbols in parentheses indicate results of comparison with rats bearing subcutaneous tumors of same age.
†Including the standard error of the mean.
‡$P = <0.05>0.01$.
§$P = <0.01$.

of the results in tables 1 and 2 demonstrated the influence of the site of the tumor on the rate and extent of alterations in the concentrations of the components of blood and serum. The shorter induction period of the intramuscular implants was presumably due to a better vascular supply. Tumors of the same weight in the earlier stages of growth (*cf.* groups B′ and C) elicited systemic effects of the same magnitude. Differences between groups with well-established tumors (*cf.* groups D and D′) were more marked, notwithstanding the similarity in the weights of the carcinomas. The identity of the protein : carbohydrate ratios in the latter 2 groups suggested that the divergences in the systemic effects were essentially quantitative.

Tissue studies.—The results of the chemical analyses of saline extracts of tumors are shown in table 3. The presence of carbohydrate-containing proteins with solubilities similar to those occurring in serum was demonstrated in the experimental carcinoma. Highly significant increases occurred only in the protein content of necrotic areas and were presumably due to the trapped blood. Tissue values were in the same range for the same gross type of tissue and were not influenced to any great extent by perfusion or the age of the tumor. The increased tissue glycoprotein : tissue protein ratio observed for the perfused, viable tissue of group F probably reflected less trapped blood in the tissue and suggested that the saline-soluble tumor-tissue proteins contained a higher percentage of bound carbohydrate than serum (tables 1 and 4).

Lyophilized serum.—The studies reported in this section (table 4) provided a comparison with the tissue studies and corroborated to a considerable degree the findings reported with fresh serum. A comparison of the serum values for the corresponding experimental groups in tables 1 and 4 indicated that the differences reported in table 1 were influenced to some extent by hemodilution.

Discussion

The Walker carcinosarcoma 256 grows rapidly following induction and is highly malignant, the death of the host occurring in 3 to 4 weeks following implantation. Alterations in the concentrations of the components of blood and serum therefore occur at a more rapid rate than is usually observed in clinical cancer or in animals with less malignant tumors.

No statistically significant changes occurred in total serum glycoprotein concentrations until the tumor constituted approximately 1 percent of the residual carcass weight. The decline in serum glycoproteins other than seromucoid is in marked contrast with the data reported for clinical cancer in which general increases in the protein-bound carbohydrates of serum have been observed (*2*, *3*).

When the seromucoid levels were subtracted from total serum glycoprotein values, the resulting glycoprotein : protein ratios declined significantly even though marked decreases had also occurred in serum protein concentrations. These changes might reflect the preferential utilization of

TABLE 3.—*Protein-bound carbohydrates in saline extracts of tumor tissue*

Group	D Nonperfused		E Nonperfused		F Perfused	
Number of samples	11		10		10	
Age of tumor (*days*)	14		21		18	
Tissue classification	Viable	Necrotic	Viable	Necrotic	Viable	Necrotic
Tissue glycoprotein (*mg. per 100 gm. dried tissue*)*	760 ± 32	890 ± 39†	730 ± 34	810 ± 43	735 ± 60	929 ± 40†
Histomucoid fraction (*mg. per 100 gm. dried tissue*)*	110 ± 6	140 ± 11†	120 ± 9	140 ± 11	126 ± 23	143 ± 10
Tissue protein (*gm. per 100 gm. dried tissue*)*	22.6 ± 0.9	28.1 ± 1.5‡	21.1 ± 0.9	25.6 ± 1.0‡	17.1 ± 1.6	28.9 ± 2.3‡
Tissue glycoprotein ÷ tissue protein × 100 (%)*	3.4 ± 0.13	3.2 ± 0.06	3.5 ± 0.09	3.2 ± 0.08†	4.3 ± 0.43	3.2 ± 0.16†
Histomucoid ÷ tissue glycoprotein × 100 (%)*	14.5 ± 1.1	15.7 ± 1.3	16.5 ± 1.7	17.3 ± 1.6	17.1 ± 3.1	15.4 ± 1.5

*Including the standard error of the mean.
†$P = <0.05 >0.01$.
‡$P = <0.01$.

TABLE 4.—*Effects of tumor growth on the protein-bound carbohydrate concentrations of lyophilized serum*

Group	A Normal	D Tumor-bearing	E Tumor-bearing
Number of animals.....	17	15	17
Age of tumor (*days*).....	—	14	21
Total serum glycoprotein (*mg. per 100 gm. dried serum*)*.....	1810 ± 21	1830 ± 70	1950 ± 52
Seromucoid fraction (*mg. per 100 gm. dried serum*)*.....	230 ± 12	540 ± 45†	640 ± 28†
Total serum protein (*gm. per 100 gm. dried serum*)*.....	70.7 ± 0.92	64.7 ± 1.31†	59.6 ± 1.31†
Total serum glycoprotein ÷ total serum protein × 100 (%)*....	2.6 ± 0.04	2.8 ± 0.09‡	3.3 ± 0.07†
(Total serum glycoprotein − seromucoid) ÷ total serum protein × 100 (%)*.....	2.2 ± 0.03	2.0 ± 0.04†	2.2 ± 0.05
Seromucoid ÷ total serum glycoprotein × 100 (%)*.....	12.7 ± 0.67	29.5 ± 1.74†	32.8 ± 0.95†

*Including the standard error of the mean.
†$P = <0.01$.
‡$P = <0.05 > 0.01$.

certain carbohydrate-containing proteins for tumor growth or their degradation to meet the increased energy demands of the tumor-bearing host (*10*).

The elevations in the levels of the seromucoid fraction were more uniform but of the same magnitude as those observed in far-advanced malignant neoplastic disease in man (*11*). No increases occurred in seromucoid concentrations, however, until the tumor was well established and had attained appreciable size. This may well account for some of the variability of results in patients with clinical carcinoma and the uniform conclusion that seromucoid determinations are of little value as a laboratory aid in the diagnosis of cancer (*11, 12*). The data also corroborate the concept proposed by Winzler (*3*) that in view of the number of protein-bound carbohydrates present in serum it is possible that different components have divergent pathologic and physiologic processes. It seems possible that the increases in the seromucoid fraction represent a systemic reponse to tissue damage (*13*), while the decreases in the other glycoproteins may be due to their utilization by the rapidly growing tumor.

The quantitative differences in blood-serum values in rats bearing subcutaneous and intramuscular implants, respectively (tables 1 and 2),

emphasized the influence of the tumor site on systemic effects. Gross differences were also noted in the carcinomas; those growing subcutaneously were more necrotic and exhibited greater liquefaction than the intramuscular implants. Many factors might be responsible for the noted variances: (*a*) greater utilization of somatic tissue as a source of nutrients by the animals that received intramuscular implants, (*b*) hemoconcentration, (*c*) different degrees of anorexia, and (*d*) release of a humoral factor from necrotic tissue that impaired serum protein and hemoglobin synthesis. No information relative to the above factors was obtained in the current study.

The presence of protein-bound carbohydrates in normal and neoplastic tissues is well established (*1*, *14*). That the tumor might be a source of the carbohydrate-containing proteins of serum has been considered by several investigators (*1*, *2*). The occurrence of fairly constant levels of protein-bound carbohydrates in saline extracts of the experimental tumor, irrespective of age, size, and perfusion, and the lack of correlation with serum concentrations, casts considerable doubt on the carcinoma as an important source of serum glycoproteins. Protein-bound carbohydrates with solubility properties similar to those of tumor tissue occur in the brain, diaphragm, heart, kidneys, liver, lungs, muscle, spleen, skin, and testes of adult, male Sprague-Dawley rats and may also represent potential sources of serum glycoproteins.[5]

Summary

Serial determinations of the effects of the growth of subcutaneous and intramuscular implants of the Walker carcinosarcoma 256 on the serum concentrations of total glycoprotein, the seromucoid fraction, and total protein and on the hemoglobin and hematocrit values of blood have been determined in adult, male Sprague-Dawley rats. Similar estimations were made on saline extracts of lyophilized serum and tumor tissue. Highly significant increases occurred in the seromucoid fraction in animals with well-established tumors coincident with a decline in other serum glycoproteins, total serum protein, hemoglobin, and hematocrit concentrations. Some of the serum and blood values for groups that received intramuscular implants differed significantly from the corresponding groups that were inoculated subcutaneously. The concentration of carbohydrate-containing proteins in saline extracts of tumor tissue was not influenced significantly by the age, size, or perfusion of the tumor.

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Cytologic Changes in the Mouse Intestine under Daily Exposure to *Gamma* Rays [1, 2]

S. LESHER, *Division of Biological Research, Argonne National Laboratory, Lemont, Illinois*

The sensitivity of the intestinal mucosa to irradiation was first described in the early reports of Warren and Whipple (*1–5*). Interest in this sensitivity has been intensified recently by cytologic (*6*) and histochemical (*7*) studies characterizing the sequence of intra- and extracellular changes after exposure to radiation and by the advancement of the concept of intestinal mode of acute radiation death by Quastler (*8–11*).

This paper reports a study of the cellular injury produced in 4 portions of the mouse intestine by continuous *gamma* irradiation at 2 dose levels (2.5 kr per day and 27 kr per day), including the histochemically detectable changes in the behavior of the nucleic acids and the periodic acid-Schiff-positive substances. Observations were continued from ½ hour after the beginning of exposure to death. Since lethality studies show that all mice exposed to continuous *gamma* irradiation at the rate of 27 kr per day die within 80 hours, while those given a dose of 2.5 kr per day do not begin to die until approximately 84 hours, it was thought of interest to determine the role of intestinal damage in causing death of the animals at the 2 levels of exposure.

Materials and Methods

Equal numbers of male and female LA F_1 mice were used in each of the 2 exposure series. The mice were 100 days of age at the time of irradiation. They were maintained 2 in a cage, separated according to sex, and allowed food and water *ad libitum*. Cages and *gamma*-exposure facilities have been described in a previous report (*12*).

The desired *gamma*-ray doses were delivered by a 1000 C Co^{60} source. Exposure was continuous except for brief interruptions to remove animals for sacrifice. The cages were placed so that the 2 series of mice received 2.5 and 27 kr per day, respectively. Mice were sacrificed at time intervals shown in table 1. Two males and 2 females were killed at each sacrifice period, and 3 portions of the small intestine and 1 segment of the colon were removed and fixed. Care was taken to remove the same section of

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[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

the intestine from each mouse: the duodenum just distal to the pyloric sphincter; the jejunum at the midpoint of the small intestine; the ileum immediately proximal to the ileocecal valve; and a section from the colon approximately 10 mm. distal to the cecum.

TABLE 1.—*Intervals at which mice were sacrificed and total dose accumulated at each interval*

2.5 kr per day		27 kr per day	
Sacrifice interval after beginning of exposure (*hours*)	Total accumulated dose (*kr*)	Sacrifice interval after beginning of exposure (*hours*)	Total accumulated dose (*kr*)
½	0. 05	½	0. 6
1	0. 1	1	1. 1
2	0. 2	1½	1. 7
4	0. 4	2	2. 3
6	0. 6	2½	2. 8
8	0. 8	3	3. 4
12	1. 3	3½	3. 9
24	2. 5	4	4. 5
36	3. 8	8	9. 0
48	5. 0	12	13. 5
60	6. 3	16	18. 0
72	7. 5	20	22. 5
84	8. 8	24	27. 0
96	10. 0	36	30. 5
108	11. 3	48	54. 0
120	12. 5	60	67. 5
		72	81. 0

The fixative was a 20:2:1 mixture of 70 percent ethyl alcohol, 20 percent neutralized formalin, and glacial acetic acid. Each section of the intestine was handled with extreme care to avoid mechanical damage to the tissues. After fixation (4 hours), tissues were washed in a graded series of ethyl alcohol concentrations of 70 percent, 80 percent, and 95 percent (2 changes of 30 minutes each), cleared in amyl acetate (2 changes of 30 minutes and 1 hour), and infiltrated with paraffin (2 changes). All sections were cut at 5 μ from blocks prefrozen overnight at $-5°$ C.

Sections were stained with Delafield's hematoxylin and eosin and van Gieson's stain. To demonstrate basophilic substances (nuclear and cytoplasmic), sections were stained in 1:1000 aqueous solution of toluidine blue buffered to *p*H 4.5 with McIlvaine's buffer. All toluidine-blue preparations were destained overnight in absolute tertiary butyl alcohol to remove the unreacted dye. To identify these basophilic substances further, control sections were incubated for 2 hours at 37° C. in 0.1 percent ribonuclease adjusted with McIlvaine's buffer to *p*H 6.8. The basophilic material removed by ribonuclease (RNase) digestion was cytoplasmic for the most part and was considered to be ribonucleic acid (RNA). For the identification of deoxyribonucleic acid (DNA), sections were stained by the Feulgen reaction.

The presence of polysaccharides was demonstrated by the periodic

acid-Schiff (PAS) reaction of McManus (*13*). The PAS reaction was used before and after diastase digestion.

The same techniques were used in the study of a large series of control animals.

Observations

Intestine of Normal Mice

Histologically, the small intestine of the mouse lacks well-defined microscopic landmarks that can be used to differentiate its 3 divisions. The most pronounced structural difference to be noted in the small intestine is the size of villi (figs. 1–3). The long, narrow duodenal villi become progressively shorter in the jejunum and the ileum. The colon, with its broad distinctive folds and complete lack of villi, is readily recognized.

At the pyloric end of the duodenum, Brunner's glands lie in the mucosal and submucosal connective tissue. Within a very short distance (less than 1.5 mm. from the pyloric sphincter in most cases) they come to lie entirely beneath the muscularis mucosae. Brunner's glands, which seldom extend beyond 10 mm. from the pyloric sphincter, empty, by numerous ducts, into the crypts of Lieberkühn near the base of the villi.

Histologically, the cells of Brunner's glands are primarily of the mucous type; they secrete a substance that is mucoid in nature and alkaline in *p*H (*14*). This alkaline secretion serves to neutralize the acidic gastric juices discharged into the small intestine. The mucoid droplets are stained intensely by PAS reagent but are unreactive to both eosin and toluidine blue.

The crypts of Lieberkühn of the small intestine are structurally similar along the entire length of the small intestine. However, the size and complexity of the crypts appear to vary directly with the surface area of the villi. For example, the long duodenal villi have the largest surface area and the crypts in this region are deep and extensively arborized. In the ileum, the length of the villi is reduced, with a consequent reduction in surface area. Paralleling this change, the ilial crypts are reduced in depth and less branched than in the duodenum.

At the base of every crypt of Lieberkühn of the small intestine lie the Paneth cells. The cytoplasmic secretory droplets of the Paneth cells give a PAS-positive reaction and stain faintly with toluidine blue at *p*H 7.0. The remaining crypt cells are concerned with the production of new cells to maintain the villar mucosa and to furnish replacements for the Paneth cells. These actively dividing cells stain intensely with toluidine blue at *p*H 4.5.

In the small intestine the villi are covered by a single layer of simple columnar epithelium. Throughout its length, the cells lining the intestinal lumen are characterized by a well-defined PAS-positive cuticular border. Goblet cells are scattered among the principal epithelial cells along the length of the villus and in the upper region of the crypts. Sections stained by the PAS method give a clear picture of the mucin-containing goblet cells. The number of goblet cells varies over a wide range at all 3 levels

of the small intestine. Counts made on sectioned material indicate that in the duodenum the number is low (approximately 1 goblet cell to every 12 principal cells) but increases in the ileum (1 to 7). These proportions of goblet cells to principal cells are only rough estimates since, according to Moe (*15*), whole mounts must be used to determine actual numbers.

In sections stained with toluidine blue at *p*H 4.5, a narrow band of basophilic granules appears above the nuclei parallel to the cuticular border. The cuticular border does not stain with toluidine blue at either *p*H 4.5 or 7.0. The oval nuclei of the epithelial cells of the villus are considerably smaller than the crypt nuclei and have less affinity for both toluidine blue and Feulgen reagent. All goblet cells are stained metachromatically by toluidine blue, with the most intense reaction in those of the ileum.

Recent experimental evidence establishes without question that most of the new cells originating in the crypts move up the sides of the villi (*16–18*). As new cells leave the crypts, the old cells at the tip are extruded. Less than 2 days from the time a cell leaves the crypt, it reaches the extrusion zone. The best estimate of the time involved is to be found in a paper by Leblond and Stevens (*18*) on the renewal of the intestinal epithelium in the albino rat. In the rat, the epithelium of the duodenal villi is renewed every 1.6 days, whereas in the jejunum and ileum it is replaced in 1.4 days. Hooper (*16*) found that it takes 1.3 days to replace the epithelium of the mouse jejunum, which suggests that the renewal rate is slower than in the rat.

The older epithelial cells near the tips of the villi (fig. 4) differ in many respects from the new cells at the base. The cytoplasm of the older cells at the villar tip is frequently vacuolated and has little affinity for either acid or basic dyes. The nuclei are small and frequently pyknotic. The cuticular border is increasingly more irregular near the villar tip and disappears entirely in the cells at the extrusion area.

Between the epithelial cells covering the villi are cells with little cytoplasm and densely stained nuclei. These are the so-called migratory lymphocytes, which come from the underlying mucosal connective tissue that forms the core of the villus. They appear to move between and not into the villar cells as suggested by Andrews (*19*). The numbers of migratory cells of the villi are quite constant and do not vary significantly throughout the length of the intestine. These cells are also found between the cells of the crypts but in considerably smaller numbers.

The mucosal and submucosal connective tissue forms a supporting framework for the villi and fills in the area between the intricately branched crypts. The connective-tissue stroma is composed of a mass of interwoven fibers and a somewhat heterogeneous cell population. A majority of these cells are fibrocytes, but they also include lymphocytes, plasma cells, and an occasional granulocyte. Lymphocytes also accumulate in submucosal nodules; in the mouse, lymph nodules are randomly scattered from the duodenum to the cecum.

The colon (fig. 5) differs greatly in structure from the small intestine.

It is composed of massive folds reinforced with a strong connective-tissue core and surrounded by thick muscular walls. No delicate villi project into the lumen. Numerous crypts open out onto the surface. These crypts are lined with cuboidal epithelial cells (figs. 6 and 7), of which a highly variable number function as mucin-producing goblet cells. Mitosis is confined to the lower region of the crypts, and the new cells furnish replacements for the cells extruded at the surface. The mitotic rate is much lower than that of the small intestine, indicating a slower turnover in the cells of the epithelium.

In the colon, goblet cells (figs. 6 and 7) are confined primarily to the crypts and, in nonirradiated mice, the number is extremely variable. These cells stain vividly with PAS and intensely metachromatically with toluidine blue. The cell population of the mucosal connective tissue of the colon is similar in diversity to the small intestine, but discrete lymph nodules are rare.

Intestine of Irradiated Mice

For the first 36 hours of continuous total-body *gamma* irradiation, either at 2.5 kr per day or at 27 kr per day, the mice appear grossly normal. They begin to lose weight at 24 hours in the 2.5 kr per day series and at 12 hours in the 27 kr per day group. By 48 hours their fur is ruffled and a majority have diarrhea. Movement of food from the stomach stops entirely at about 60 hours with 2.5 kr per day and at 48 hours with 27 kr per day. The stomach becomes impacted with food material, while increasing amounts of blood and mucin accumulate in the remainder of the lower intestinal tract.

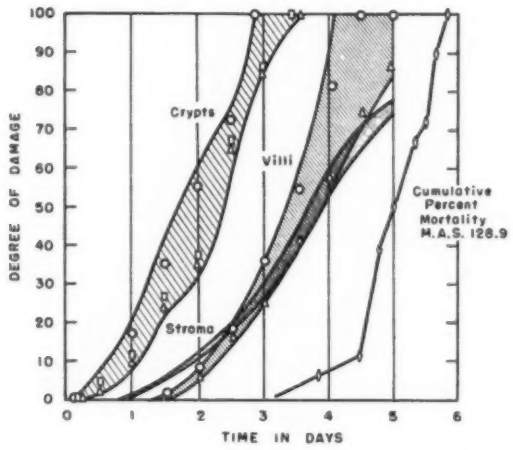
The 2.5 kr per Day Series

Mice exposed to continuous *gamma* irradiation at 2.5 kr per day have a mean aftersurvival of 128.9 hours, with a maximum survival of approximately 142 hours. Their death is attributable largely to intestinal damage following the loss, to a variable degree, of the epithelium of the villi (denuding) of the small intestine. Damage produced by the irradiation appears first in the duodenum; however, the entire small intestine is soon involved.

The crypts are extremely susceptible to irradiation, as shown graphically in text-figure 1, and are completely destroyed along the entire length of the small intestine by 96 hours. At that time denuding of the duodenal villi (fig. 15) is in progress and by 120 hours (fig. 19) it is completed. Most of the mice die before the jejunum (fig. 20) and ileum (fig. 21) undergo denuding.

Damage to the colon never reaches the same extent as that in the small intestine. The goblet cells of the crypts discharge most of their mucin by 120 hours, but neither the crypts (fig. 22) nor the surface of the folds are denuded.

Crypts.—The first cytologic changes appear in the nuclei of the cells that form the walls of the crypts. The number of mitotic figures in the



TEXT-FIGURE 1.—Damage shown by the principal cell populations of the small intestine of mice exposed to continuous *gamma* irradiation at 2.5 kr per day. The range of individual variability of each cell population is represented by the cross-hatched or shaded areas. After the first day, the extent of damage to the duodenum (○) far exceeds that shown by the jejunum (□) and ileum (△). Data for cumulative percent mortality were furnished by Sacher *et al.* (*20*).

duodenal crypt cells is noticeably reduced by the end of the first half hour. Mitosis is completely inhibited after 4 hours of irradiation [total accumulated dose (TAD) 0.4 kr] in the duodenum, whereas it takes 6 hours to stop mitosis in the jejunum (TAD 0.6 kr) and 8 hours in the ileum (TAD 0.8 kr).

The crypt-cell nuclei show an early increase in size. The walls of the cells near the base of the villi become indistinct; thereafter the nuclei appear to clump together into multinucleated cells. Basophilia, as shown by basic staining with toluidine blue (*p*H 4.5), begins to decrease at 12 hours and reaches a very low level at 48 hours before crypts are denuded. In the terminal stages, from 96 hours to the time the last samples are taken—120 hours, there is a further reduction in basophilia to the point where very little stainable material remains. Intensity of the Feulgen reaction shows a similar decrease.

The breakdown in the crypts follows a definite pattern. By the end of 48 hours the crypts (figs. 8, 10) are fragmented and totally disorganized. The cells in the walls of the crypts are now separated from the cells at the base of the villi and also from the Paneth cells at the base of the crypts. These isolated crypt-wall cells and the Paneth cells are pushed together by the edematous connective tissue to form small, isolated cell nests. The fully differentiated Paneth cells, like the epithelial cells covering the villi, are quite resistant to irradiation.

The cell nests disintegrate very rapidly. By 72 hours most cell nests and crypt fragments (figs. 12, 15, 19) disappear from the duodenum; however, up to 96 hours, many crypt fragments are found in the ileum (figs. 13, 14,

17). The Paneth cells, though severely damaged and cut off from the intestinal lumen by connective-tissue stroma, are the last of the crypt components to completely disappear. These last Paneth-cell remnants still give a faint PAS-positive reaction.

The crypt cells of the colon are much more resistant to irradiation. Damage is not noticeable until 60 hours. By 84 hours the nuclei of the crypt cells become conspicuously enlarged and the outer cuticular membrane of the basal cells begins to break up. By 96 hours most of the crypt cells (fig. 18) lining the walls, as well as those at the neck of the crypts, have discharged their mucin. After the loss of mucin and the destruction of the cytoplasmic outer border, all that remains is a narrow protoplasmic layer covering the flattened nuclei lying along the basement membrane.

The breakdown of the crypts of the colon usually starts in the cells in the neck of the crypt. Nearer the base of the crypts the cells still contain mucin. Some of the nuclei of the crypt-neck cells and in the mucosal cells at the surface of the folds are swollen and distorted. Stainability of the cytoplasm and nucleus is considerably reduced. By 120 hours most of the crypts are damaged. Approximately one fourth of the crypts (fig. 22) are severely damaged, and a large percentage of the remaining crypts have degenerated to a low-cuboidal, almost squamous-type, lining.

Villi.—The mucosal membrane of the villi shows very little change until after 48 hours, although a few cells at the extrusion zone fall out within the first 24 hours. Lesions thus produced are closed off quickly (fig. 9) without damage to the remainder of the villi. Since no new cells come up from the crypts, the villi become progressively shorter. The stainability of the cells adjacent to the extrusion zone is low. The nuclei are small, distorted, and pyknotic.

By 48 hours nuclei of the epithelial cells in the proximal region of the villi (fig. 8) became swollen. The cell boundaries are irregular and incomplete. By 60 hours about one fourth of the duodenal villi show open lesions at the extrusion zone, and the connective-tissue stroma has been pushed back by the accumulation of edematous fluids at the tips of the villi. A high percentage of the villar tips (fig. 11) are edematous.

At 72 hours the villi are obviously shorter and highly irregular. The stainability of the villar cells (fig. 11) is low: The cytoplasm gives a very faint reaction with eosin and fast green and the nucleus gives an equally weak reaction with Feulgen reagent. The cuticular border also undergoes considerable change, staining faintly with eosin and to an even lesser extent, with PAS.

At 96 hours the epithelium of the duodenum (fig. 15) begins to slough off the villi, which by this time are reduced to approximately one fifth their normal length. In addition to the shortening effect, adjacent villi fuse to form low-lying mounds of distorted cells. The number of villi in a cross section is less than half the number found in a nonirradiated control. The remaining epithelial cells of the duodenal villi show severe damage. Arterioles, venules, and capillaries are prominent but contain little blood. By 120 hours the duodenum is completely denuded (fig. 19).

Damage to (*cf.* figs. 15, 16) the jejunum and ileum lags about 12 hours behind that of the duodenum. At 120 hours the low-lying villi of the jejunum and ileum are still covered with a severely damaged but intact epithelium (figs. 20, 21).

In the colon the epithelium is normal at 72 hours. Twelve hours later the nuclei and cells appear swollen. Some nuclei (fig. 18) reach several times normal size. Very few lesions can be detected along the surface of the folds. All nuclei show a drastic reduction in size from 96 to 120 hours (fig. 25). Distorted and pyknotic nuclei are quite common. In spite of these cellular changes the mucosal covering of the folds remains intact up to the death of the animal.

Goblet cells.—In the duodenum there is no significant change in the number of goblet cells. This is not unexpected, since the damage to both the crypts and villi is extremely heavy and takes effect early. The remainder of the small intestine shows a real increase in the number of goblet cells during the first 36 hours. This is particularly true of the ileum, where the number rises rapidly (figs. 28, 30, 32), reaching a level of 25 percent of the total cell number at 36 hours. This level is maintained up to 72 hours, after which the number declines rapidly to a few abnormal goblet cells at the time of death.

In the colon the number of goblet cells increases (figs. 29, 31, 33) to a peak at 48 hours. At this time the crypts of the colon show only slight microscopic damage; however, almost every crypt cell has become a goblet cell (fig. 31). The cells at the neck of the crypts contain the greatest amount of mucin. Secretion of mucin in the crypts of the colon begins in the heavily laden neck cells at approximately 72 hours. At 96 hours cells in the upper half of the crypts are devoid of mucin (fig. 18), and by 108 hours the goblet cells at the bottom of the crypts begin to discharge their mucin. By 120 hours the crypt cells (fig. 33) contain very little mucin, and, as a result, the outer cell border is narrow and fragmented (fig. 22).

Connective-tissue stroma and lymphatic tissue.—Changes in the connective-tissue stroma are difficult to evaluate; however, the cells normally found within the network of fibers are susceptible to irradiation. Of these, the lymphocytes are by far the most numerous and the most sensitive. The destruction of lymphocytes in the connective-tissue stroma begins very early. By 2 hours debris from disintegrating cells is interspersed throughout the lamina propria.

By 48 hours the connective-tissue stroma is reduced to connective-tissue fibers and a few fibrocytes. All lymphocytes have been destroyed and the cellular debris eliminated. The connective tissue that forms the core of the villi has been pushed back by the accumulated fluid at the villar tips to become part of the submucosal layer. The expanding submucosal connective tissue fills in the lesions left by the destruction of the crypts.

In the lymphatic tissue the breakdown in lymphocytes also begins at 2 hours. By 12 hours very few lymphocytes are found in the nodules,

and every nodule (fig. 27) is a mass of cell debris. An examination of the spleen and thymus of these animals reveals that the same condition exists in other lymphoid organs. The number of cell fragments increases rapidly from the time of their appearance, at 2 hours, and reaches a peak at 12 hours, after which the number decreases rapidly and disappears after 36 hours. These changes correlate very closely with the fall in lymphocyte count in the peripheral blood (*21*).

The migratory lymphocytes found between the mucosal cells of the small intestine show a very rapid increase in number during the first 2 hours of irradiation. They gradually decrease in number after 4 hours and disappear completely by 48 hours. The migratory-cell picture is essentially the same in all portions of the small intestine. Many of the migratory cells become pyknotic and undergo fragmentation. The extracellular chromatin fragments disappear shortly after the migratory cells disappear.

The 27 kr per Day Series

The mice exposed to 27 kr per day continuous *gamma* irradiation have a mean aftersurvival of 67.2 hours, and all are dead by 80 hours. The crypts of the small intestine are seriously damaged and many are completely destroyed. However, the mice die before denuding of the villi takes place, hence death is not due primarily to intestinal damage (*see* text-fig. 2).

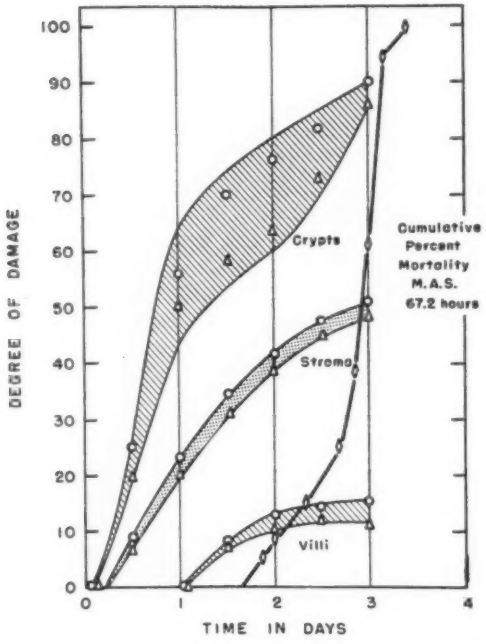
Crypts.—The first animals of this series are sacrificed after ½ hour of irradiation (TAD 0.56 kr). Already mitosis is completely inhibited along the entire intestine, large and small. Almost all the crypt-cell nuclei are enlarged at 1 hour. By 4 hours the nuclei begin to lose some of their contents and shrink. Many nuclei become shriveled and pyknotic; however, some rupture, and spherical basophilic chromatin fragments become common inclusions of the cytoplasm. These fragments stain intensely with toluidine blue (*p*H 4.5) and contain a Feulgen-positive component.

At all levels of the small intestine, crypts are rapidly breaking up at the end of the first 24 hours. Three of every 4 crypts are highly disorganized. Most of the wall cells literally disintegrate and do not form into cell nests as they do in the 2.5 kr per day group.

The cells at the neck of the crypts immediately adjacent to the base of the villi appear to fuse and close off the intestinal lumen from the damaged crypts. By 48 hours the wall cells are almost completely destroyed. The connective-tissue stroma in the crypt region is frequently filled with chromosome fragments. The Paneth cells, which show less damage, clump into cell nests. The nuclei of the neck cells become extremely large and highly distorted. In most cases their cell walls are no longer distinguishable, and multinucleated cell masses are common in this intervillar region.

By 72 hours all but a few crypts (fig. 24) are destroyed, and only an occasional clump of dead cells remains in the submucosal connective tissue.

Villi.—Damage to the epithelium of the villi never reaches the same proportions as in the 2.5 kr per day group. Even the mucosa of the



TEXT-FIGURE 2.—Damage to the principal cell populations following 27 kr per day irradiation. In this series the degree of damage is essentially the same at all levels of the small intestine with the exception of the duodenum (○) where damage is slightly more pronounced than that of the jejunum and ileum [since the jejunum is essentially the same as the ileum the figure is used to designate the remainder of the small intestine (△)]. Data for cumulative percentage mortality were furnished by Sacher *et al.* (*20*).

duodenal villi (fig. 23) shows much less damage at 72 hours. Only a few cytologic changes distinguish the irradiated from the nonirradiated; *e.g.*, there are enlarged cells at the base of the villi, a region of distorted dead or nearly dead cells as the extrusion zone reaches farther down the sides of the villi, and an over-all decrease in stainability of the epithelial cells.

The villi maintain a length comparable to those of the nonirradiated mouse intestine. Apparently few cells are extruded from the tips of the villi, and lesions at the extrusion zone are quite rare. The migratory lymphocytes reach a peak at 2½ hours, then decline rapidly; after 12 hours they are no longer observed. The goblet cells, both in the crypts and in the villi of the ileum and the colon, increase in number between 1½ and 24 hours and then decrease to a small number at 72 hours.

Connective-tissue stroma and lymphatic tissue.—Degenerative changes begin in the connective-tissue stroma at about 8 hours. The cellularity is noticeably reduced by 24 hours, and the fibrous network appears sparse by 36 hours. In the lymphatic tissue, degeneration begins at once. The cellular fragments produced by destruction of lymphocytes appear first

in the peripheral region of the nodules; after 4 hours (fig. 26) the lymph nodules are crowded with debris, and few intact lymphocytes are found. The breakdown of lymphocytes passes a peak by 8 hours, and all lymphocytes and cellular debris have been eliminated by 16 hours.

In the colon (fig. 26), the changes are primarily in the crypt cells. The crypts do not disintegrate as in the small intestine; in fact, for the most part, they retain their characteristic structure. The cells in the crypts give up almost all of their mucin, and, as a consequence, the lumen border and outer layer of cytoplasm is disrupted. The mucosal cells covering the folds show very little damage.

An attempt was made to evaluate quantitatively the degree of cumulative damage and to correlate the sequence of changes produced in the duodenum, jejunum, and ileum of the 2 series of irradiated mice. The determinations involved a careful examination of all crypts, villi, and connective tissue in 5 cross sections in each division of the small intestines of 4 mice at each time period listed in table 1. In the crypts the damaged cells were counted and the percentage per cross section was calculated. The evaluation of the villi was based on a comparison of the average length of all villi in the intestines of the irradiated animals with the average length of villi in similar cross sections from 10 nonirradiated controls. In addition, the number of degenerative epithelial cells of the villi were related to the number of apparently undamaged cells. Changes in the connective-tissue stroma were only relative since they represented only the changes in cellularity and density of fiber concentration estimated from a comparison of the tissues from irradiated and nonirradiated mice. In both text-figures the broad shaded areas emphasize the differences in the degree of damage at the 3 levels of the small intestine. They indicate that there is a progressive decrease in the degree of damage, which is maximal in the proximal region of the duodenum and reaches its lowest level in the distal sector of the ileum.

Discussion

Following acute single-dose exposures ranging from 1 to 10 kr, the survival time of mice was about 3 to 5 days (*11*), and death was preceded or accompanied by loss of the epithelial lining of the small intestine. This characteristic syndrome is the "acute intestinal radiation death syndrome," in which damage to the intestine plays the dominant role. In this study the mice that received 2.5 kr per day died in 3.5 to 5.5 days and, in all those examined, denuding of the duodenum had started or was completed. On the other hand, the mice that received 27 kr per day died between 2 and 3.5 days with their intestinal epithelium intact. In the 2.5 kr per day group, loss of body fluids through the extensive duodenal lesions was probably one of the primary causes of death, while in the 27 kr per day series, damage to the intestine probably was not the critical factor.

After single-dose exposure to acute irradiation at comparable levels, the initial damage was followed by recovery, the degree of which was

dose-dependent. In the intestinal crypts of mice that received continuous acute *gamma* irradiation, cell division was inhibited and remained inhibited, thus making recovery impossible. Although the pattern of degenerative changes that occurs in the duodenum, jejunum, ileum, and colon of mice following continuous acute *gamma* irradiation was similar in the early phases to that following acute single-dose exposures, the fact that there was no recovery phase to deal with made it possible to evaluate better 1) the sequence of changes in the destruction of the crypts, 2) the life span of the mucosal epithelium of the villi, and 3) differences in susceptibility of the 3 levels of the small intestine and colon.

Destruction of the crypts at both 2.5 and 27 kr per day followed the same pattern and proceeded at approximately the same rate (text-figs. 1 and 2). The walls of the crypts proved to be more sensitive to irradiation than either the Paneth cells at the base of the crypts or the so-called neck cells at the top of the crypts. The neck cells, which seldom divide, were actually already determined cells that were differentiating into cells to repopulate the villi, and, as a result, they were more resistant to irradiation. The order of decreasing sensitivity of the crypt cells appeared to be wall cells, neck cells, and Paneth cells.

In both the 2.5 and 27 kr per day series, most of the cells of the epithelium of the villi lived well beyond their normal life expectancy. If Hooper (*16*) is correct in her estimate that it takes almost 2 days for cells to move from the base of the villus to the extrusion zone, then the cells near the base of the villus in figure 10 were already 2 days of age and normally would already have been extruded. Similarly, the cells near the tip of the villus in figure 11 were 2 days of age at the time of irradiation; 72 hours later, they were 5 days of age but still had not been extruded. However, in spite of their increased life span, these cells still functioned as a protective covering for the underlying mucosal connective tissue and the lymph and blood vessels therein. The ability of the epithelial cells to maintain their position on the villus beyond their normal life span may actually serve as a compensatory protective mechanism. Hence, when mitosis is stopped in the crypts, the intestine may actually have a safety margin of some 3 to 3.5 days before denuding exposes the mucosal tissues. This extension of time may be sufficient to permit the crypts to recover and give rise to new cells for the repopulation of the villar covering. This same safety factor might be expected to operate in any situation in which mitosis in the crypts is stopped.

The epithelium of the villi of mice exposed to 27 kr per day showed less radiation damage at the end of 3 days than that of mice receiving 2.5 kr per day for the same period. The villi of the former were near normal length, while the villi of the 2.5 kr per day series were about 20 percent shorter. In the 2.5 kr per day series there was a continuous loss of cells at the extrusion zone from the beginning of irradiation, with the rate increasing at 2.5 days, to the death of the animal. In the 27 kr per day series, very few, if any, cells were extruded. In addition, the

staining of both the nucleus and cytoplasm was more intense in the 27 kr per day group. The principal, if not the only, difference in the behavior of the intestinal cells of the 2 series was in the time at which mitosis was stopped. In the 2.5 kr per day group, mitosis continued 4 to 8 hours, while in the 27 kr per day series it stopped in the first half hour, and all cells were essentially frozen in position. It is possible that the extrusion of cells from the tip of the villus is dependent upon the pushing up of new cells from the crypts and that in the mice receiving 27 kr per day the mucosal cells of the intestinal villi were able to retain their position because of the immediate inhibition of mitosis. Since, on a microscopic level, damage to the intestinal villi of mice receiving 27 kr per day was minimal, there was no apparent regional difference in sensitivity. In the 2.5 kr per day series there were considerable differences, which could be resolved cytologically. The order of sensitivity appeared to be duodenum, jejunum, ileum, and colon.

The nucleic acid concentration of the crypt and villar cells, as shown by staining with basophilic toluidine blue and by the Feulgen reaction, was decidedly reduced after *gamma* irradiation. Other workers have also reported changes in nucleic acid metabolism following exposure to ionizing radiation (*6*, *7*, *22*). The nucleic acid content reached a very low level in both the nuclei and cytoplasm of the mucosal cells of the villi by 3 days after exposure to either 2.5 or 27 kr per day. Lewis, Quastler, and Svihla (*23*) presented evidence that seems to indicate a movement of nucleic acid particles from the nucleus to the cytoplasm within 10 minutes following irradiation with X rays.

There is considerable disagreement about the reaction of mucous cells to ionizing radiation. Some investigators (*24*) believe that exposure to high doses depletes the goblet cell of mucin or that the synthesis of mucin may be completely stopped (*25*). Friedman (*26*) found that in the rat, goblet cells continued to produce mucin after high single doses of X rays. Montagna and Wilson (*6*) found that in mice X-irradiated with 1000 r, the number of goblet cells decreased, after 48 hours, from 11 to 6 percent in the duodenum. In mice exposed to continuous *gamma* irradiation in the acute range, this was quite true of the duodenum. However, since entire crypts were also destroyed or severely damaged, the decrease in goblet cells was a direct result of crypt destruction and not of a special sensitivity of goblet cells.

In both the ileum and the colon, where damage was much less severe, there was an actual increase in goblet-cell numbers. In the colon, after 48 hours of continuous irradiation, almost every crypt cell contained mucin. Other irritants also stimulate excessive production of mucin by the crypt cells of the colon (*27*). This nonspecific reaction suggests that in the colon all crypt cells are potentially capable of synthesizing mucin.

Summary

A microscopic study was made of the cytologic and histochemical changes in the intestine of the mouse after continuous total-body *gamma* irradiation at 2 dose levels, 2.5 kr per day and 27 kr per day.

In the 2.5 kr per day series, mitosis stopped in the crypts of the duodenum after 4 hours, in the jejunum after 6 hours, and in the ileum after 8 hours. Crypts were completely destroyed in the duodenum by approximately 3 days and in the jejunum and in ileum from 6 to 12 hours later. The mitotically active cells of the crypt walls were by far the most sensitive, followed by the cells in the neck immediately adjacent to the base of the villi, and then the Paneth cells at the bottom of the crypts. The cells covering the villi were surprisingly resistant to irradiation, but as cells were extruded from the tip of the villi, the villi became progressively shorter. After the first day, this was particularly true of the duodenum, which was completely denuded in 120 hours. The mice died before the jejunum and ileum were entirely denuded. In the colon neither the crypts nor the mucosal membrane covering the folds were denuded. Goblet cells in the ileum and colon increased in number, up to 48 hours. In the crypts of the colon every cell appeared to have the potential ability to synthesize mucin. Mice of this series died after 3½ to 5½ days of irradiation, following partial or complete denuding of the duodenum; death was due to the "acute intestinal death syndrome."

In the 27 kr per day series, mitosis stopped in all regions of the intestine within the first half hour. The crypts were essentially destroyed by 3 days in the small intestine but showed little damage in the colon. The villi, on the other hand, maintained a nearly normal length even in the duodenum. The cells showed cytologic changes, *e.g.*, nuclear swelling followed by shrinkage and pyknosis, but denuding of the villi did not take place. Denuding might be hastened by movement of cells following the formation of new cells in the crypts. The mice receiving 27 kr per day died after 2 to 3½ days of irradiation, with intestinal mucosal epithelium of the villi intact. Intestinal damage probably played a minor role as the cause of death at this dose level. The dominating factor or factors may be determined when other tissues from the same animals are analyzed.

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PLATE 25

The 3 figures in this plate are taken from sections of the small intestine of a non-irradiated mouse to show the size relationship that exists between the crypts and villi at each level and the size difference between levels. The duodenal section is approximately 8 mm. from the pyloric sphincter but in this mouse is beyond Brunner's glands. These figures are composite photographs. Sections are stained with hematoxylin and eosin. × 350

FIGURE 1.—The duodenum.

FIGURE 2.—The jejunum.

FIGURE 3.—The ileum.

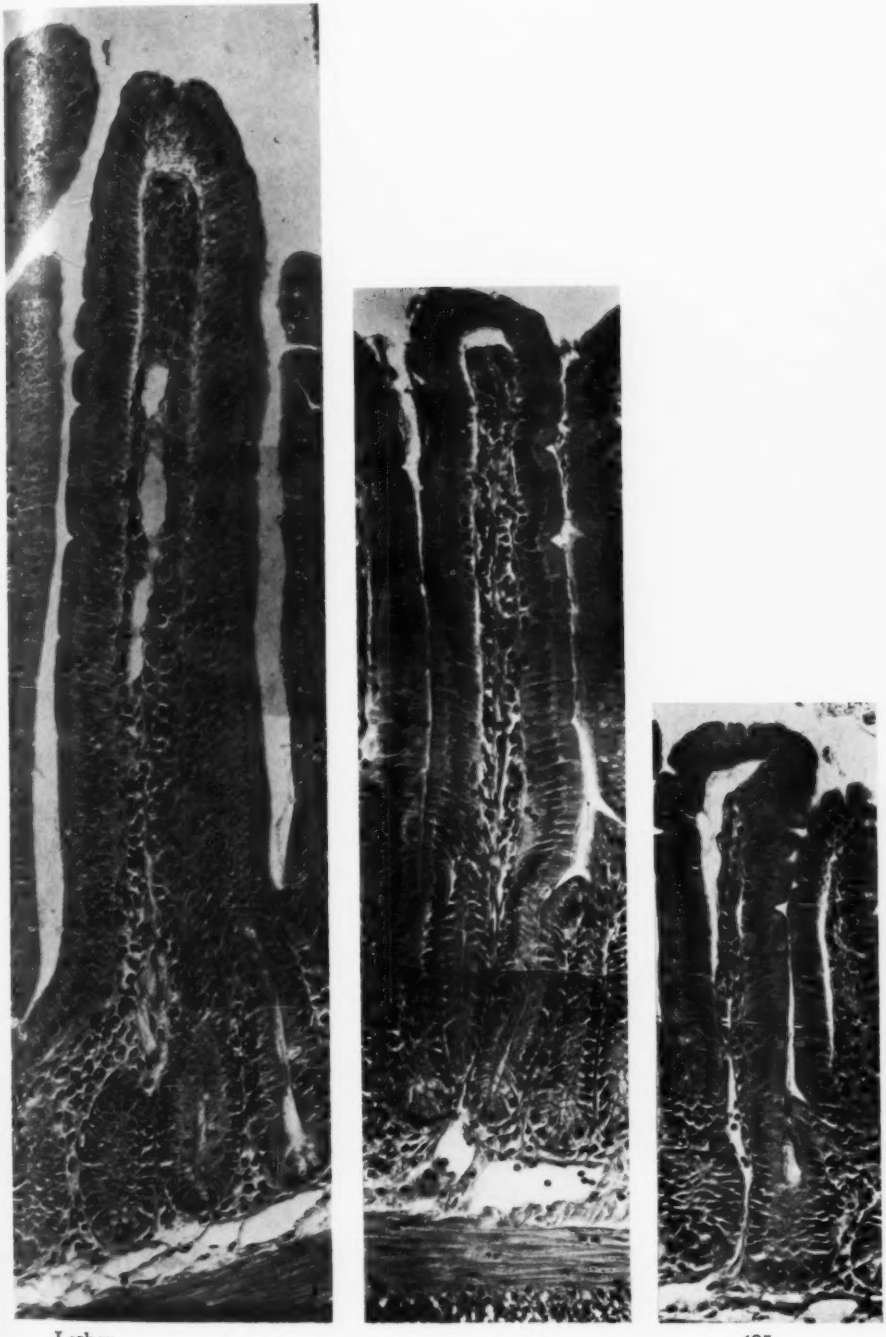

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PLATE 26

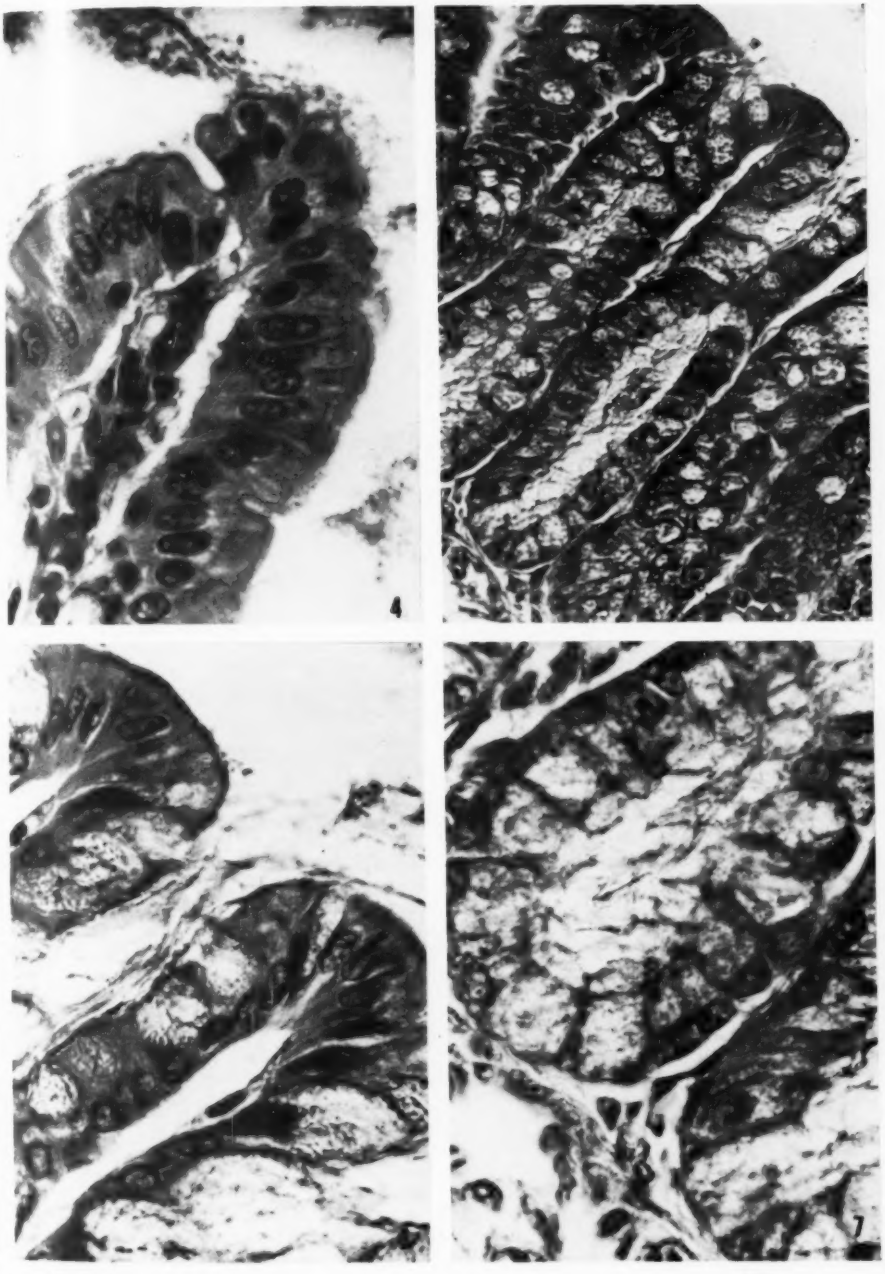
All figures in this plate are taken from sections of tissues, stained with hematoxylin and eosin, from nonirradiated mice.

FIGURE 4.—Tip of duodenal villus showing extrusion zone. Notice the diffused nature of the cuticular border of the cells near the extrusion zone and the pyknotic nuclei of cells about to be extruded. × 960

FIGURE 5.—Section of mouse colon showing the crypt and surface mucosal cells. Goblet cells are numerous throughout the crypts but seldom extend beyond the neck to the surface of the folds of the colon. × 420

FIGURE 6.—Higher magnification of upper crypt region and mucosal surface. Notice collapsed goblet cells on or near the surface of the fold. Goblet cells in the upper crypts are spherical. × 960

FIGURE 7.—Higher magnification of the crypt shown in *center* of figure 5. The entire base is lined with goblet cells. Nuclei are slightly compressed against basement membrane. The lumen of the crypt contains strands of faintly eosinophilic material (cytoplasmic) which are forced out with the discharged mucin. × 960



All figures in plates 27, 28, and 29 and figures 20, 21, and 22 in plate 30 are taken from sections of intestine from mice exposed to continuous *gamma* irradiation at the rate of 2.5 kr per day and stained with hematoxylin and eosin. Figure 23 in plate 30 and figures 24, 25, and 26 in plate 31 are taken from sections of intestine from mice exposed to 27 kr per day. × 420

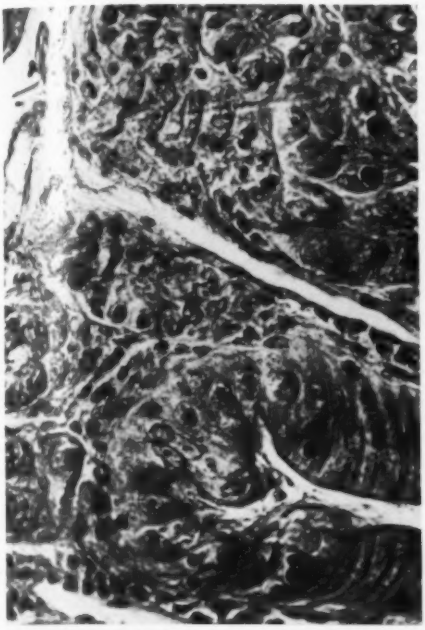
PLATE 27

FIGURE 8.—Section of duodenum at base of villus after 36 hours of irradiation, total accumulated dose (TAD) 3.75 kr. Crypts are highly disorganized and are beginning to break up into small cell nests. Nuclei of cells at the base of the villus are swollen, cell boundaries are indistinct, and necrotic cell fragments are common.

FIGURE 9.—Duodenal villi after 48 hours of continuous irradiation, TAD 5 kr. The cuticular border is highly irregular. There are pyknotic nuclei near the tips of the villi, and open lesions at the extrusion zone are common.

FIGURE 10.—Base of duodenal villi from the same section pictured in figure 9. Notice the jumbled mass of cells at the base of the crypt in the *lower-right corner*. Crypts do not exist as organized units.

FIGURE 11.—Duodenal villi after 72 hours of continuous irradiation, TAD 7.5 kr. Villi are edematous and nuclei are shrunken and distorted and the cytoplasm is low in stainability. Mucosal connective tissue has been pushed from villar tip, and the base of mucosal cells is irregular and torn



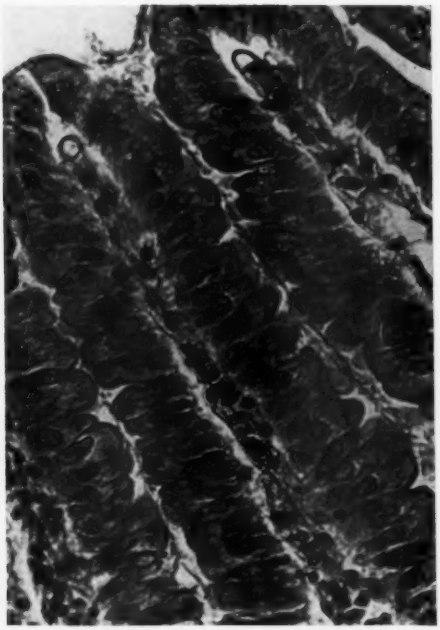


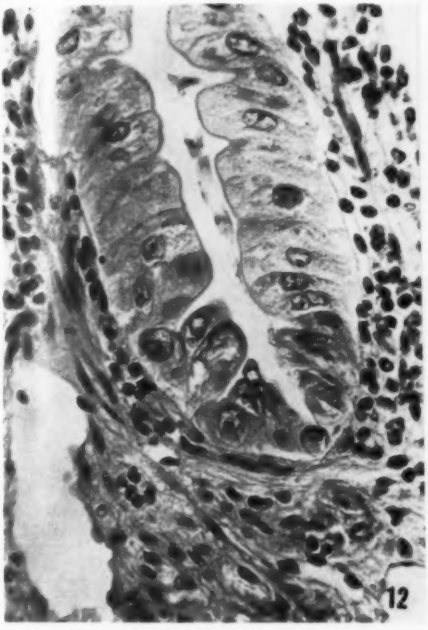
PLATE 28

FIGURE 12.—Base of duodenal villi from the same section shown in figure 11. Numerous lesions mark the sites of former crypts, *e.g., lower-right corner*. Such lesions fill in rapidly by the expansion of mucosal and submucosal connective-tissue stroma. Many nuclei contain unusually large nucleoli (2 such nuclei can be seen at the base of the villus). × 420

FIGURE 13.—Ileal villi after 72 hours of continuous irradiation, TAD 7.5 kr. The villi are reduced in length by extensive loss of cells at extrusion zone. The remaining cells expand to close lesions created by loss of cells. Mucosal cells are changed to a thick, squamous-type epithelium. Nuclei vary from very small to extremely large; however, all are distorted in shape. Syncytial masses are common. × 420

FIGURE 14.—Submucosal area from the same ileal section shown in figure 13. Disintegrating crypts are cut off and isolated from the villi. Nuclei are in various stages of degeneration: vesicular, pyknotic, fragmented, autolyzed, and necrotic. × 420

FIGURE 15.—Duodenum after 96 hours of continuous irradiation, TAD 10 kr. Crypts are completely destroyed, and villi are reduced to low mounds of connective tissue covered by vacuolated, essentially dead cells. × 420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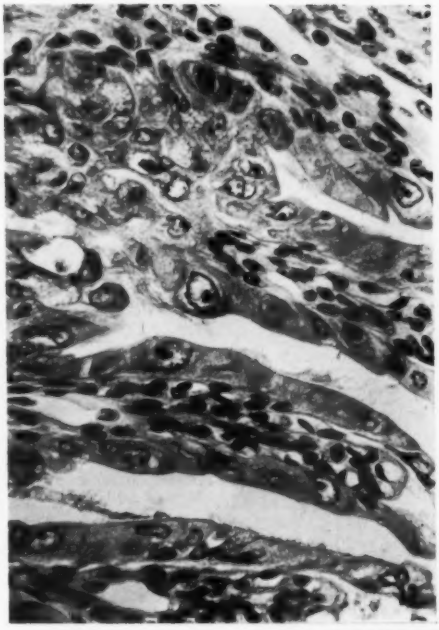

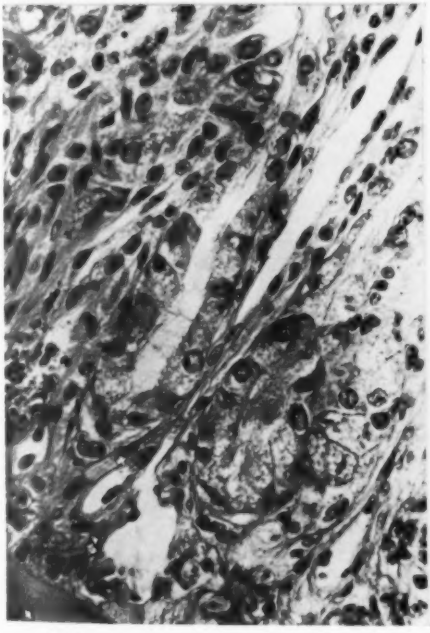

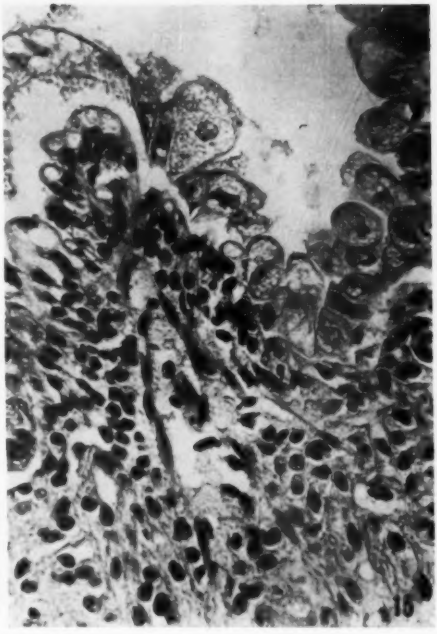
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PLATE 29

FIGURE 16.—Ileal villi after 96 hours of continuous irradiation, TAD 10 kr. Villi are short and cells are large, with distorted nuclei. × 420

FIGURE 17.—Crypt region from the same ileal section shown in figure 16. Crypts are denuded or reduced to a few flattened cells (2 to 4 to a field). × 420

FIGURE 18.—Colon after 96 hours of continuous irradiation, TAD 10 kr. The mucosal epithelium covering the surface between crypts is intact. A majority of the nuclei are swollen. The crypts are still heavily laden with goblet cells, which have discharged most of their mucin. The nuclei are flattened against the basement membrane. × 420

FIGURE 19.—Duodenum after 120 hours of continuous irradiation, TAD 12.5 kr. *Arrow* points to denuded villi. There are no crypts. Numerous lesions mark the sites of former crypts in the submucosal connective tissue. × 420

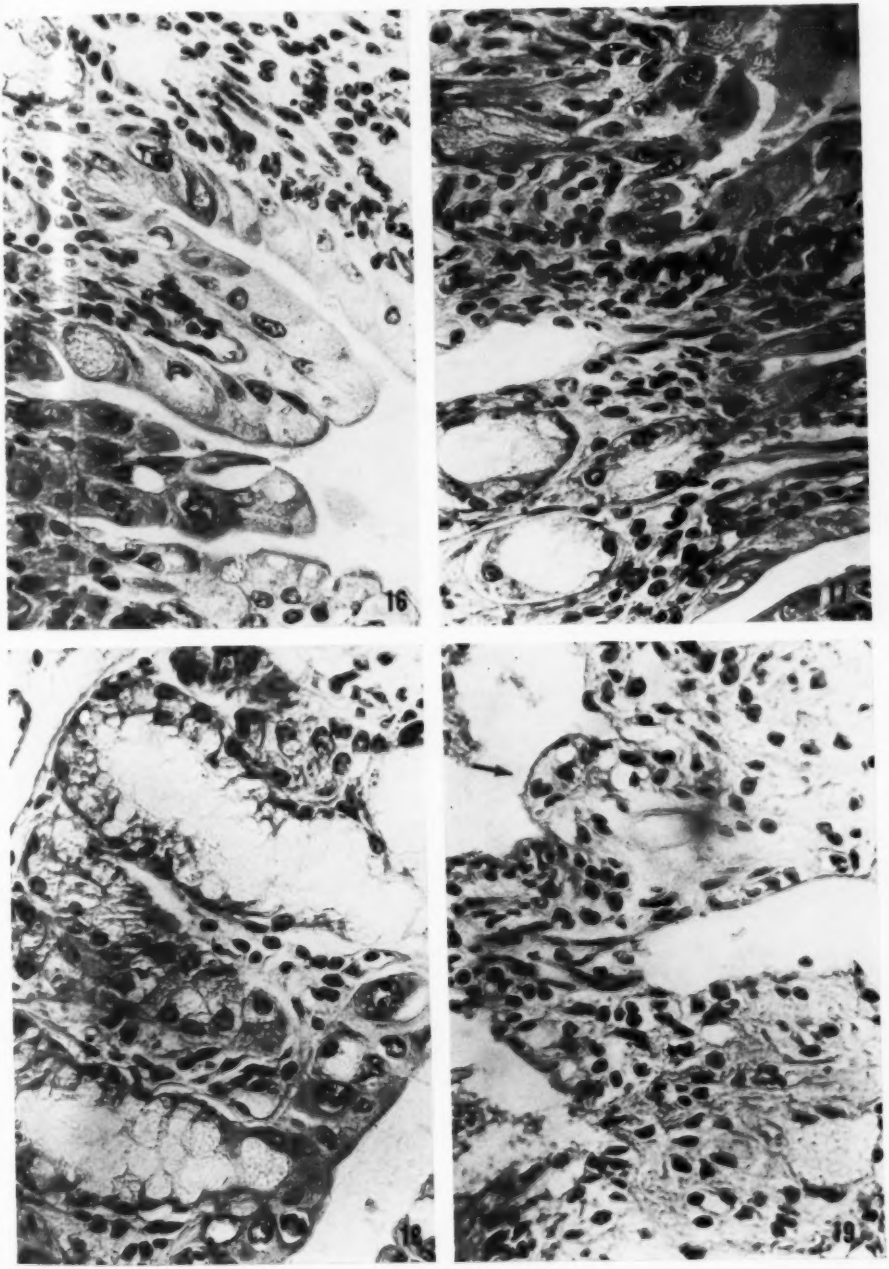
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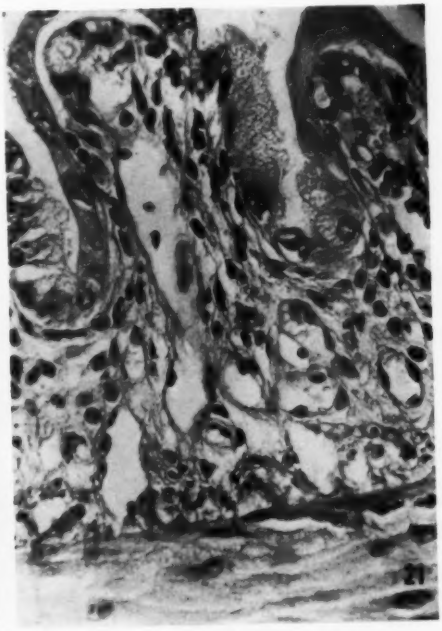
PLATE 30

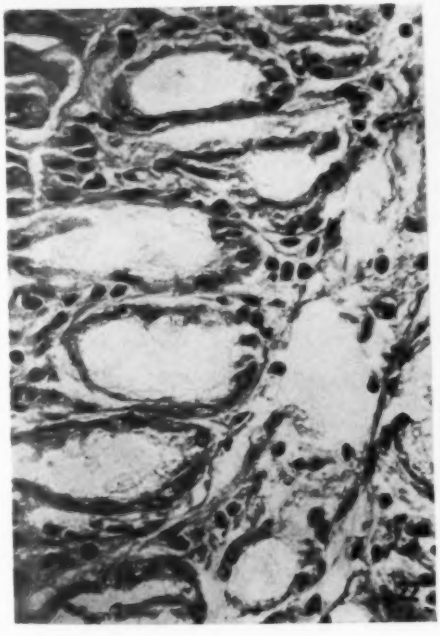
FIGURE 20.—Jejunum after 120 hours of continuous irradiation, TAD 12.5 kr. Mucosae are severely damaged and cells obviously dead; however, the villi are not denuded. There are no crypts and connective-tissue stroma are scant. × 420

FIGURE 21.—Ileum following 120 hours of irradiation, TAD 12.5 kr. Mucosae similar in condition to jejunum. Crypts are completely denuded but not filled in by the expanding submucosal connective tissue. × 420

FIGURE 22.—Colon after 120 hours of irradiation. Mucosal membrane (not shown) is still intact but severely damaged. The goblet cells of the crypts (which in this mouse probably made up over 95 percent of the crypt cells) have discharged their mucin. Crypt cells are simple squamous in type. × 420

FIGURE 23.—Duodenum after 72 hours of continuous *gamma* irradiation at a rate of 27 kr per day, TAD 81.0 kr. The villar covering is intact; however, the nuclei are smaller and inclined towards pyknosis and the cuticular border is extremely irregular. Compare this figure to figure 11 from a mouse that received a TAD of 7.5 kr after 72 hours of irradiation. × 420





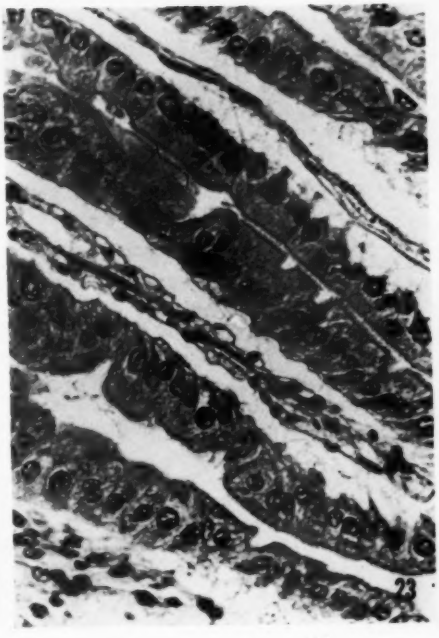


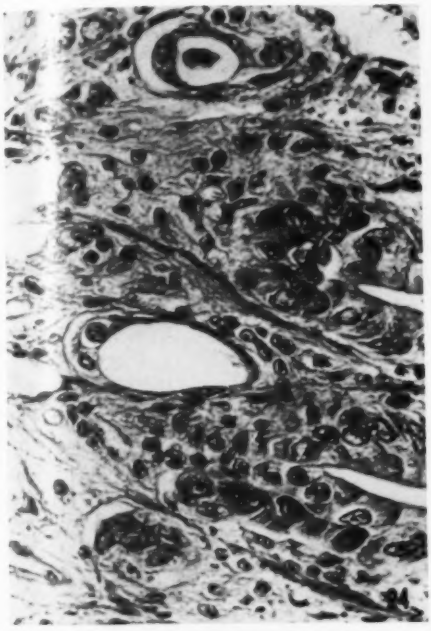
PLATE 31

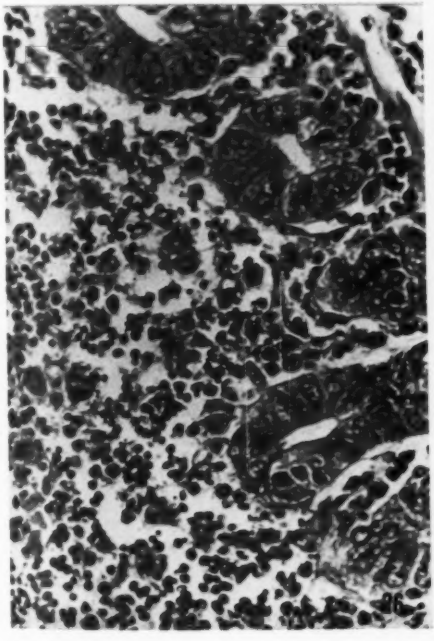
FIGURE 24.—Duodenal crypts lying at the base of the villi shown in figure 23. The neck region of the crypts has been severely damaged. Cell walls are ruptured and masses of distorted nuclei are clumped together. Only a few isolated crypt fragments remain, and in these the cells are reduced to a few cytoplasmic threads and flattened nuclei. × 420

FIGURE 25.—The colon after 72 hours of continuous irradiation at 27 kr per day, TAD 81.0 kr. Mucosal membrane of the fold surface is intact. Nuclei of cells located in crypt walls are enlarged. The goblet-cell number was not as high as found in the 2.5 kr per day series after a similar period of exposure. × 420

FIGURE 26.—Lymph nodule in ileum of mouse after 3½ hours of irradiation at 27 kr per day, TAD 3.4 kr. Clumps of fragmented lymphocytes fill the lymph nodule, and few intact lymphocytes are to be found. × 125

FIGURE 27.—Lymph nodule in ileum of mouse after 12 hours of irradiation at 2.5 kr per day, TAD 1.25 kr. Concentration of lymphocyte fragments similar to that found in figure 30. × 420





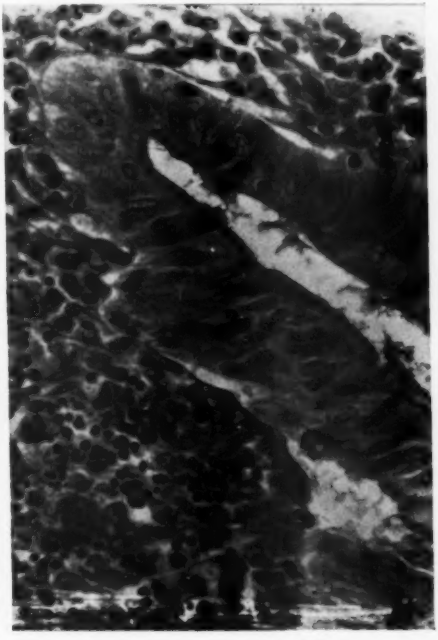


PLATE 32

Change in goblet-cell number in ileum and colon after exposure to continuous *gamma* irradiation at 2.5 kr per day. Severe and early damage to duodenum eliminates any possibility of demonstrating a change in goblet-cell number. Sections stained by the PAS reaction without counterstaining. × 125

FIGURE 28.—Ileum after ½ hour of irradiation, TAD 0.05 kr.

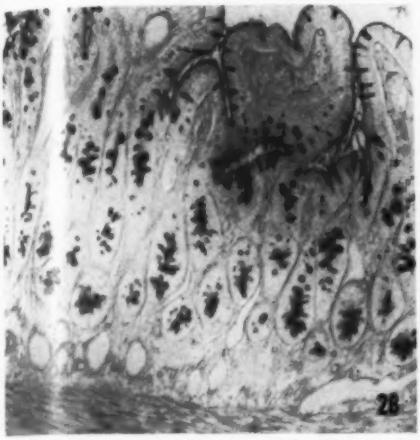
FIGURE 29.—Colon after same exposure as in figure 28.

FIGURE 30.—Ileum after 24 hours of irradiation, TAD 2.5 kr.

FIGURE 31.—Colon after same exposure as in figure 30.

FIGURE 32.—Ileum after 72 hours of irradiation, TAD 7.5 kr.

FIGURE 33.—Colon after same exposure as in figure 32.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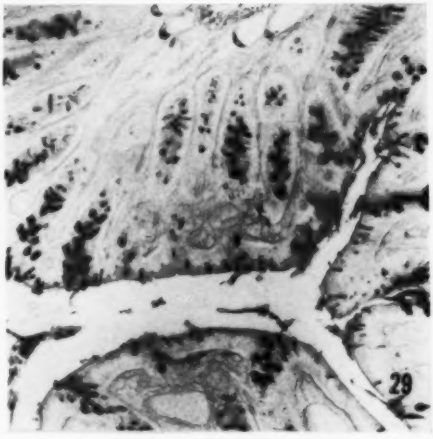
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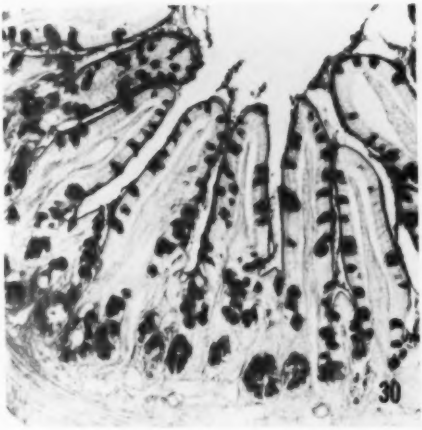
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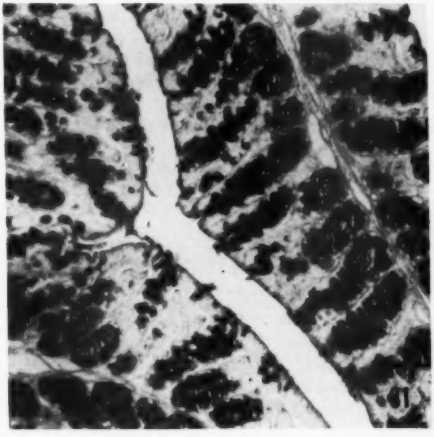
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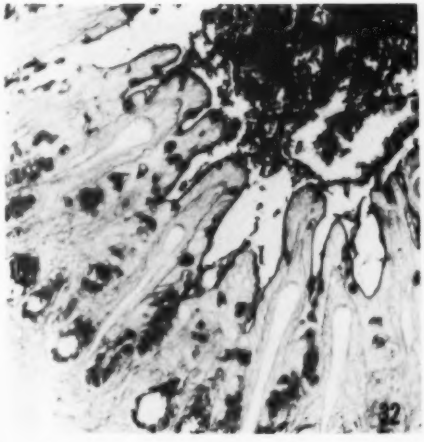
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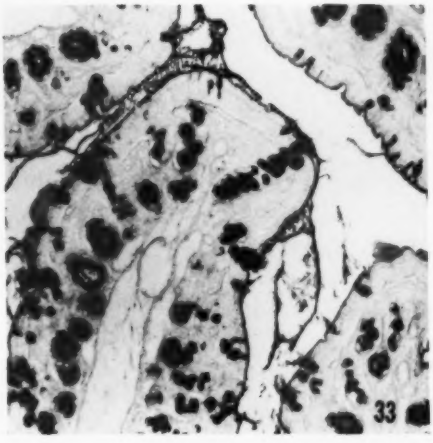
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Histology and Cytochemistry of Human Skin. XIII. The Blood Supply of the Hair Follicle [1,2]

WILLIAM MONTAGNA *and* RICHARD A. ELLIS,
Arnold Biological Laboratory, Brown University, Providence, Rhode Island

The patterns of capillary networks around the hair follicles of the rat and sheep have been investigated (*1*, *2*); those around the hair follicles in human skin are relatively unknown, since the only effective way of making capillary nets visible is to inject them with opaque substances. Because this is no longer an obstacle, the endothelial cells of human capillaries can be demonstrated selectively by applying azo-dye techniques for alkaline phosphatase to frozen sections of skin. This method has all of the advantages of injection techniques and none of the disadvantages. Collapsed or blocked capillaries show up as clearly as capillaries that are patent. It will be shown that the capillary network around human hair follicles is similar to that of the rat and sheep and the configuration of the capillaries around active follicles is entirely different from that surrounding quiescent hair follicles.

Materials and Methods

The pieces of skin for this study were secured at surgery and autopsy from every region of the body. Materials from cadavers not over 4 hours old are adequate for this purpose. Over 100 specimens were obtained from 28 subjects, 3 to 75 years of age. All the specimens were presumably of normal skin. For convenience, all tissues were stored in dry ice from 1 to 5 days. Small strips of skin 3 to 5 mm. thick were fixed for 4 hours in 10 percent neutral, unbuffered formalin. Frozen sections were cut about 40 μ thick; thicker or thinner sections were cut according to our needs. The sections were thoroughly washed in distilled water and then treated with the azo-dye technique of Gomori (*3*) for alkaline phosphatase. Sodium α-naphthyl acid phosphate was used for the substrate and fast blue RR for the coupling dye. To prevent precipitation during incubation, the sections were transferred to 2 or 3 fresh changes of the substrate-dye mixture. The color developed in about 10 minutes; the sections were washed in distilled water and stored for at least 24 hours in 10 percent

[1] Received for publication May 27, 1957.
[2] This investigation was supported in part by research grant RG 2125 C7 from the National Institutes of Health, U. S. Public Health Service.

formalin. Gas bubbles escaping from freshly prepared sections are all released during this time. The sections were washed in distilled water, floated onto slides, and mounted in glycerin jelly. To show some of the histological details, we stained a few sections lightly in Mayer's carmalum. Thick sections require 2 or 3 days to clear.[3]

Observations

The technique used in this study reveals the endothelium of the capillaries almost specifically; the endothelium of arterioles and venules is very weakly reactive. In thick sections, where entire capillary limbs can be traced to their parent arteriole, the endothelium is practically unreactive in the arteriole and in the first part of the capillary, but as the capillaries reach their final loops and networks, the endothelial cells show a progressive increase in reactivity (fig. 1). This technique is particularly useful for our study, because, aside from the capillaries and sweat glands, human skin has very little demonstrable alkaline phosphatase.

We have found it convenient to store tissues in dry ice. Even to store them for a week does no appreciable harm to the reaction. Comparison of tissues processed immediately after removal at surgery or autopsy with those stored in dry ice shows very little diminution of enzyme activity in the capillaries and no distortion in the capillary tufts.

The results we have obtained from fresh autopsy material are similar to those from surgical specimens. These descriptions, therefore, are a summation of all the materials we have studied. Although the vascular patterns of the skin undergo dramatic aging changes, which we are now studying, none seem to be reflected in the blood vessels of healthy hair follicles.

Hair follicles from the different regions of the body have different cycles of growth. Those in the scalp, for instance, have very long periods of growth and brief periods of quiescence; those from the torso and appendages grow for a brief period but rest for a long time. There are some structural differences between these different follicles, but they are of a minor nature. The over-all structural pattern in hair follicles is similar and the metamorphoses from growing to quiescent and back again to growing are identical, whether in the follicles of the scalp, the face, the axilla, or the scrotum. It is possible to generalize and then compare the follicles from one region to those from others as we have in figures 2 and 3 where a follicle from the scalp is compared with one from the scrotum. These follicles have been chosen for their photogenicity, and they also embody all the features described here.

Hair follicles are surrounded by a continuous plexus of capillaries, and the pattern of the plexus is different in active and in quiescent follicles and will be described separately (figs. 2, 3).

The lower third of active follicles, from the base to just above the bulb, is enveloped by a rich vascular plexus composed of long, more or less

[3] We wish to thank Shelly Weisman for her excellent technical assistance.

parallel vessels connected by cross-shunts (fig. 2). Most of the parallel vessels emerge from the papilla pore and go straight up the follicle (fig. 4); the cross-shunts are oriented somewhat horizontally, but they are often tortuous and have secondary shunts that form a latticework pattern (fig. 4). The bore of the longitudinal vessels is always larger than that of the cross-shunts, and for this reason they are assumed to be terminal arterioles. From just above the bulb, up to the level of the sebaceous glands, most of the cross-shunts drop out, and the follicle is surrounded only by the palisade of parallel longitudinal vessels (figs. 2, 5). In thick preparations these can be traced from their emergence from the papilla pore all the way up to the upper levels of the follicles. At the level of the sebaceous glands, the vessels once again form a network that envelops both the glands and the pilary canal (fig. 6). The parallel vessels lose their identity around the pilary canal and terminate in a loosely woven network that extends to the surface of the skin. Loops of capillaries form a vascular ring around the orifice of the follicle under the epidermis; this collar is continuous with both the vascular loops of the epidermis and the network around the follicle. Sebaceous glands are richly vascularized (fig. 8); baskets of capillaries surround the acini and follow all of their contours.

The dermal papilla at the base of each follicle contains a large tuft of capillaries in its center. This occupies a much larger part of the papilla than is ordinarily realized. These capillaries extend to the walls of the inner side of the follicle and are often practically in contact with the wall. Arterioles and capillaries enter the capillary tuft of the papilla from below (fig. 7), and several vessels emerge from the tuft at the papilla pore (fig. 4); they flow around the lip of the pore and rise straight up the sides of the bulb. The vascular system of each follicle is a continuous unit from the dermal papilla to the area around the infundibulum, including the plexus around the sebaceous glands. Although some arterioles and capillaries supply the capillary plexus of the follicle from the sides, the principal vessels come from below by way of the dermal papilla (fig. 7).

Quiescent hair follicles have no bulb, and they are much shorter than active ones. The dermal papilla rests free against a pedicle of cells at the base of the epidermal capsule that surrounds the club hair. A dense palisade of capillaries connected by short, transverse vessels surrounds the free dermal papilla and extends for a distance below it (fig. 3). A few straight vessels from this bundle rise along the sides of the follicle, go up to the level of the sebaceous glands, and form a network like the one described in active follicles. A few cross-shunts form a sparse network at the base of the epithelial capsule around the club hair.

The follicles of vellus hairs in the body skin or in the bald scalp are surrounded by very simple capillary systems. A few capillaries surround the lower part of the follicle, but no vessels penetrate the dermal papilla, which is much more reactive for alkaline phosphatase than that of large follicles. The enormous sebaceous glands associated with these follicles are normally highly vascularized.

There are many follicles intermediate in size from those of vellus hairs to those of small overhairs. The vascular patterns of papillae of these follicles show a graded transition from one type to the other. There is also an inverse relation between the amount of phosphatase activity and the fullness of the capillary plexus.

Discussion

Using this technique, we are studying the vascular patterns of the skin in every important region of the human body. In subsequent papers we will elucidate the capillary networks in other cutaneous appendages and those underneath the epidermis.

The vascular pattern of human hair follicles is similar to that of the follicles of the rat and the sheep, demonstrated by injecting these animals with India ink (*1*, *2*). A complex network of capillaries is found around the bulb and a less complex one farther up around the pilosebaceous canal; when the follicles are quiescent, a bundle of capillaries is located at their base. During the transition from active state to quiescence, the bulb atrophies, and the base of the follicle moves upward in the dermis and partially out of the network that surrounds it. The dermal papilla also recedes upward and slips away from its central capillary tufts. Only a part of these capillary nets seems to atrophy; most of the vascular material collapses and remains at the base of the resting follicle as a tangled bundle in contact with the free dermal papilla. Since only the lower part of a follicle degenerates when it becomes quiescent, the palisade of parallel vessels in the middle third remains unchanged. Higher up, around the sebaceous gland and the infundibulum, the plexus also remains intact. When a quiescent follicle becomes active again, the new bulb plows its way through the bundle of capillaries and grows inside it.

The capillary networks from any part of a follicle and its sebaceous glands form a continuous system, and each vascular bed is a unit. Since the pilosebaceous system functions as a unit, the vascular mechanism may be responsible for this integration or at least shares the responsibility.

Nearly the entire lower third of the follicle consists of the bulb; in its lower end the cells of the matrix proliferate constantly, move up, and form the hair and the inner root sheath. In its upper part the cells that have come from the matrix grow many times their original volume and synthesize fibrous proteins; the final phases of keratin formation take place in the keratogenous zone in the upper limit of the bulb. At this level of the follicle, the cells of the outer root sheath contain more glycogen than those in other levels (*4*, *5*) and more esterases and β-glucuronidase (*6*, *7*). The follicle is surrounded at this level by a glassy membrane that is thicker than at any other level. These characteristics suggest that the outer root sheath at this level is a very active tissue. Ryder (*8*) has given the strongest evidence for this by showing in the follicle of sheep that only 6 minutes after the injection of cystine labeled with S^{35}, a strong accumulation of radioactive particles can be recovered first around the general region of the keratogenous zone. This is the region of the follicle

surrounded by the densest capillary networks in the sheep, the rat (*1*), and man. The region of the upper part of the bulb is unquestionably an important site of exchange.

Exchange must also take place through the dermal papilla. The amount of vascular tissue in a dermal papilla is dependent on its size: the wider it is, the larger the capillary tufts it contains (*2*). The dermal papillae of active human follicles are very wide and contain large quantities of endothelium. During periods of quiescence, either the tissue of the papilla disengages itself from the capillary tuft by flowing away from it or the capillaries atrophy. Such large numbers of capillaries in the dermal papilla strengthen the belief (*4*) that the bulk of the papilla cells remains relatively the same during periods of follicle activity and quiescence and that the changes in the size of the papilla are largely due to the increase and decrease in the amount of capillary tissue and to the increase and decrease in the size of the papilla cells.

Summary

1) The azo-dye techniques for alkaline phosphatase effectively demonstrate the endothelium of the terminal capillaries in frozen sections of skin.

2) Active hair follicles have a rich plexus of capillaries around the bulb; a sparser plexus in the upper part of the follicle also envelops the sebaceous glands. Straight vessels along the middle, less vascularized part of the follicle connect the lower with the upper plexus.

3) The dermal papilla of active follicles contains a large capillary tuft from which vessels run up the sides of the follicle plexus.

4) The capillary system around each follicle is a continuous unit.

5) Quiescent follicles with no bulb slip out of the lower capillary plexus, which remains collapsed at the base of the follicle. The dermal papilla either flows away from the capillary tuft, or it atrophies.

6) The follicles of lanugo hairs are surrounded by a very simple capillary network. The dermal papilla contains no capillaries.

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PLATE 33

FIGURE 1..—An arteriole, beginning at the *arrow* at *bottom* of page, terminates into 2 capillary plexuses around coils of sweat glands in *upper part* of figure. Observe that the vessel shows practically no phosphatase reaction (*arrow*), but the reactivity becomes increasingly intense toward the terminal capillaries. Biopsy specimen from the skin of the scrotum of a man 23 years old. × 200

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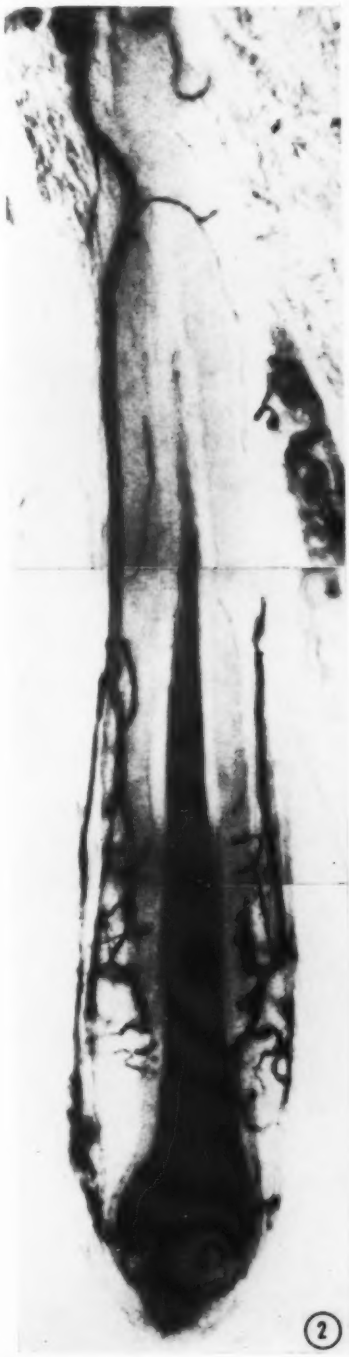
PLATE 34

FIGURE 2.—Composite photograph of the lower two thirds of an active hair follicle from the scalp of a man 41 years old. The plexus of capillaries extends to the upper level of the keratogenous zone, where the diameter of the hair shaft constricts suddenly. The middle part of the follicle, in the *upper part* of the figure, contains only a few parallel vessels. × 55

FIGURE 3.—Composite photograph of a quiescent hair follicle from the scrotum of a man 33 years old. Contrast the total length of this follicle with that in figure 2. Observe particularly the dense bundle of vessels at the base of the dermal papilla. × 55


2

3

PLATE 35

FIGURE 4.—The capillary plexus around the bulb is shown clearly in this preparation. *Arrow* in *upper left* corner points to one of the parallel vessels that emerges from the papilla pore. From the scalp of a man 33 years old. × 100

FIGURE 5.—Parallel vessels around the middle of a follicle in a biopsy specimen from the scalp of a man 30 years old. *Upper end* of the plexus around the bulb is shown at the left of the figure. × 100

FIGURE 6.—The capillary plexus around the upper end of a follicle from the scalp of a man 33 years old. Observe the capillary loops under the epidermis on the right of the figure. × 125

4

5

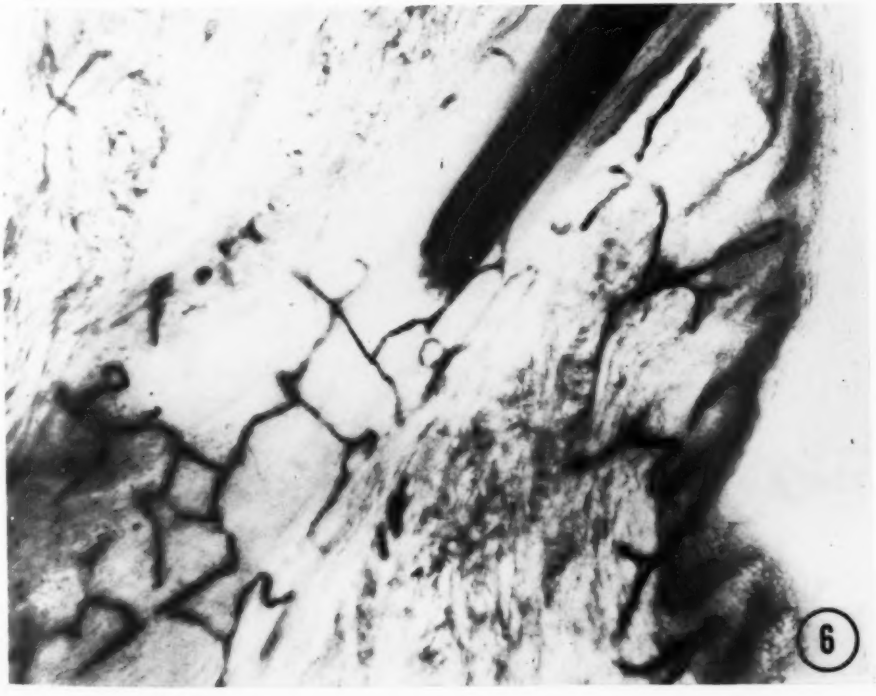
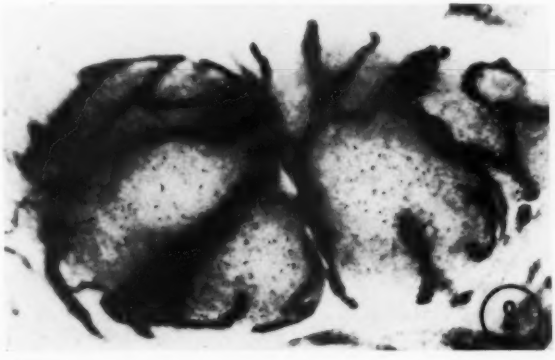

6

PLATE 36

FIGURE 7.—Sagittal section through the bulb of a follicle from the scalp of a man 30 years old, showing the capillary tuft particularly well. *Arrow* points to an incoming vessel. This preparation was lightly counterstained with carmalum. × 240

FIGURE 8.—Two sebaceous glands completely enwrapped by strongly reactive capillaries. From the skin of the scrotum of a man 23 years old. × 125



ANNOUNCEMENT

Seventh International Cancer Congress

Applications and Travel Allotments

The Seventh International Cancer Congress will be held in London, England, July 6 to 12, 1958, under the sponsorship of the International Union Against Cancer. Scientists and physicians are invited to submit papers on experimental or clinical aspects of cancer or on cancer control. These papers should represent new contributions to knowledge and must not have been published or reported previously. The deadline for registration without late fee and also for submission of papers is January 1, 1958. A preliminary program and application forms for registration, submission of papers, etc., are available on request to either of the following:

1. The Secretary General, Seventh International Cancer Congress, 45 Lincoln's Inn Fields, London, W. C. 2, England.
2. Dr. Harold F. Dorn, Secretary General, Union Internationale Contre Le Cancer, National Institutes of Health, Bethesda 14, Maryland.

Travel allotments of approximately $530 each will be available to a limited number of scientists and physicians residing in the U. S. A. who may require such assistance. This amount is expected to provide payment of: special 15-day overseas round-trip air tourist fares, a 6-day per diem allowance, and reimbursement for registration fee. Investigators who have been invited to participate in one of the symposia prior to or following the Congress may apply for additional funds.

Applications for travel allotments should be submitted in sextuplet in the form of letters giving information on age, training, publications in cancer or related fields, academic or professional title, and institutional affiliation. These letters should be countersigned by the department director or administrative officer. Applicants for travel funds who are submitting papers for presentation at the Congress must include six copies of an abstract (not to exceed 250 words) of each such paper. An applicant not planning to present a paper at the Congress should include six copies of an abstract of major current investigative work. Such letters and abstracts must be submitted in sextuplet before January 1, 1958, to the Chairman, U. S. A. National Committee on the International Union Against Cancer, 2101 Constitution Avenue, N. W., Washington 25, D. C.

Applications for assistance toward travel expenses are entirely separate from the applications for registration for the Congress and for the submission of papers to the Program Committee. All applicants will be responsible for their own passports, visas, registration, travel arrangements, and hotel reservations.

U. S. GOVERNMENT PRINTING OFFICE: 1957

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited. Color transparencies should be accompanied by a set of black-and-white photographs.

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